

THE CYTOCHROMES c OF CAMPYLOBACTER
SPUTORUM SUBSPECIES MUCOSALIS

by

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Thesis presented for the Degree of
Doctor of Philosophy

Department of Biochemistry
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August 1982



Declaration

In accordance with the Regulations of the University of Edinburgh, the work presented in this thesis - apart from the assistance acknowledged is my own. Part of this work has been published in a preliminary communication.

Acknowledgement

I am greatly indebted to Professor G S Boyd for the training I received and for the permission to carry out research in his Department.

I would like to thank Dr G W Pettigrew and Dr J L Leaver for active supervision, criticism and guidance. I greatly benefited from their advice, encouragement and discussions.

I am grateful to Dr R P Ambler of the Department of Molecular Biology in whose laboratory amino acid sequence determination was carried out; to Mrs M Daniel for help and advice in operating the automatic sequenator; and to Dr David Apps for advice concerning the synthesis of 6-aminohexyl sepharose.

My gratitude extends to those members of the teaching and technical staff of the Department of Biochemistry, University of Edinburgh - Dr Gordon Atkins, Mrs Celia Goodhew, Mrs Ishbel Jack, Mrs S Seilman, Miss Fiona Leitch and Mrs Leslie Clunas (and family) - whose encouragement, and friendliness has made a 5 years sojourn in Scotland a more enjoyable and less painful process than it would have otherwise been.

This work was partly supported by a research grant from the Wellcome Foundation to Dr J L Leaver and Dr G W Pettigrew; while blood-agar plates on which the organism Campylobacter sputorum subspecies mucosalis was grown, were kindly prepared by the Department of Bacteriology, the Royal (Dick) Veterinary College, University of Edinburgh. Financial support to myself and my family was kindly provided as a scholarship by the University of Geziera (Sudan).

I must finally thank Miss Helen Scott for professionally executing the typing of this thesis.

Abstract

Campylobacter sputorum subspecies mucosalis, the causative organism of porcine intestinal adenomatosis, will grow microaerophillically in the presence of hydrogen as an electron donor, and under these conditions contains a high complement of c-type cytochromes. The cytochromes c in a crude extract of the organism were examined by redox potentiometry. The redox potentiometric data were consistent with the presence of at least two low midpoint potential and two high midpoint potential cytochromes c. Furthermore the prediction could be made that one of the high midpoint potential cytochromes contains an asymmetric α -peak.

A high midpoint potential cytochrome c (c_{553}) was isolated and then characterized with respect to its spectrum, midpoint potential, amino acid composition and N-terminal sequence. The pure cytochrome was shown to exhibit a midpoint potential close to that predicted from titration of the crude preparations and to possess a markedly asymmetric α -peak. The cytochrome exhibits similarities to a number of high midpoint potential low spin cytochromes c and has been compared to these. Partial isolation and characterization of three low midpoint potential low spin cytochromes c was possible. These cytochromes (the cytochromes c_{552}) show no similarity to other low potential cytochromes c reported in the literature with respect to spectrum and molecular weights and are apparently unique to the organism.

The presence of both low midpoint and high midpoint potential cytochromes c has so far been reported for only a few organisms, including members of the genus Campylobacter. In the light of the redox potentiometric data, the electron transport chain of Campylobacter sputorum subspecies mucosalis has been compared to those of other well studied organisms.

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CHAPTER I
INTRODUCTION

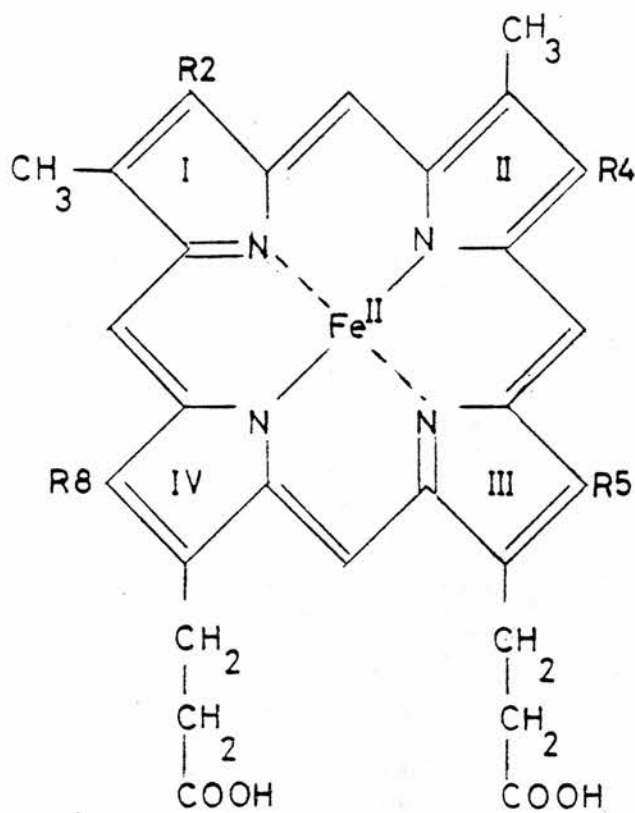
Introduction

Biological energy conservation, which takes place through membrane bound electron transport systems, almost invariably involves the participation of cytochromes. The present study is mainly concerned with the cytochromes c of a bacterial electron transport chain - that of Campylobacter sputorum subspecies mucosalis. In that context the following paragraphs will give a brief review of the cytochromes c followed by a section on the best studied bacterial electron transport systems. Finally the electron transport systems of members of the genus Campylobacter are briefly discussed and the aims of this work described.

A. The Cytochromes c

The cytochromes have been defined as haemoproteins which are involved in oxidation reduction reactions by virtue of reversible valency changes in their haem iron. On the basis of substituent groups on the haem moiety, fig. 1, the cytochromes are classified into 3 groups: cytochromes a, b, and c; while a fourth group, the cytochromes d, contain a dihydroporphyrin (chlorin) iron as prosthetic group. The position of the α -band of the pyridine ferrohaemochrome in alkali serves to distinguish these haemoproteins. The history, classification and properties of the cytochromes have been reviewed by Lemberg and Barrett 1973.

The term cytochrome c designates a large number of haemoproteins which have been identified in the mitochondria of higher animals and plants, as well as in bacteria. They have been extensively studied due to their wide occurrence and their relative ease of isolation, purification and characterization. While mitochondrial cytochromes c form a relatively homogeneous group, bacterial cytochromes c vary greatly in physicochemical properties such as size, isoelectric

Fig. 1: Structure of Cytochromes' Haem Groups

Haem	R2	R4	R5	R8
a	$\begin{array}{c} \text{OH} \\ \\ -\text{CH}-\text{CH}_2 \\ \\ (\text{CH}_2-\text{CH}_2-\text{CH}-\text{CH}_2)_3-\text{H} \end{array}$	$-\text{CH}=\text{CH}_2$	—	$-\text{CHO}$
b	$-\text{CH}=\text{CH}_2$	$-\text{CH}=\text{CH}_2$	$-\text{CH}_3$	$-\text{CH}_3$
c	$\begin{array}{c} -\text{CH}-\text{CH}_3 \\ \\ \text{S-PROTEIN} \end{array}$	$\begin{array}{c} -\text{CH}-\text{CH}_3 \\ \\ \text{S-PROTEIN} \end{array}$	$-\text{CH}_3$	$-\text{CH}_3$

points, redox potentials and number of haem groups. In general however the cytochromes c fall into 3 main groups:

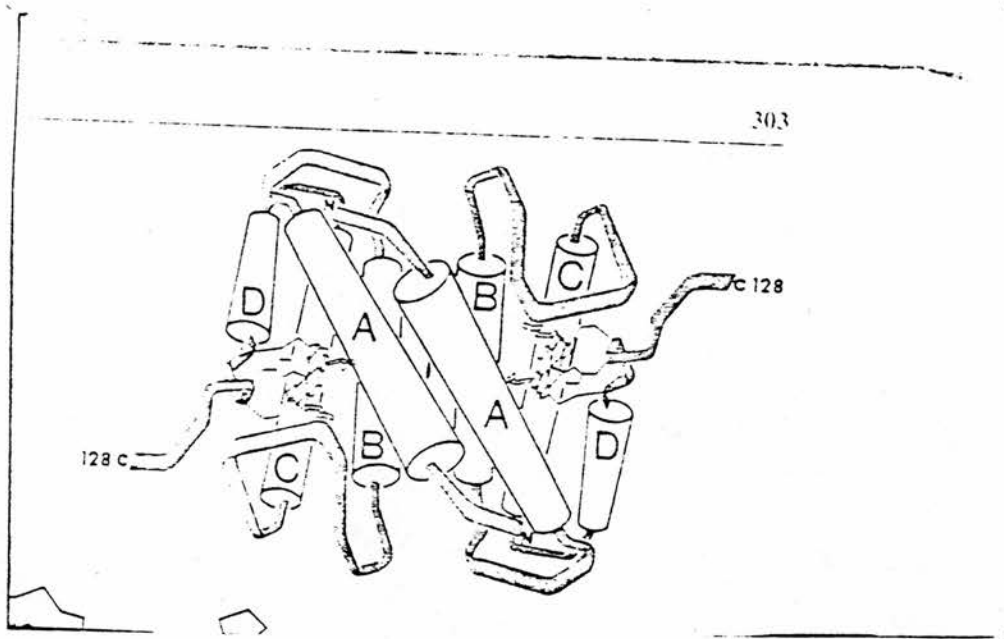
- (i) low spin high potential cytochromes with haem attachment near the N-terminus;
- (ii) high spin cytochromes with haem attachment near the C terminus (c' cytochromes);
- (iii) multihaem low potential cytochromes (c_3 cytochromes).

The second and third groups can be differentiated from each other and from the first group by their absorption spectra. The high spin cytochromes - the cytochromes c' - have been reviewed by Bartsch 1978; the tertiary structure of a member of this class has been elucidated by Weber et al. 1980. The multihaem low potential cytochromes - the cytochromes c_3 of the sulphate reducing bacteria - have been reviewed by Der Vartanian et al. 1974. Haser et al. 1979 have determined the tertiary structure of cytochrome c_3 from D. desulfuricans strain Norway. The tertiary structures of the cytochromes c' , and the cytochromes c_3 and their absorption spectra are shown in figs. 2, 3 and 4.

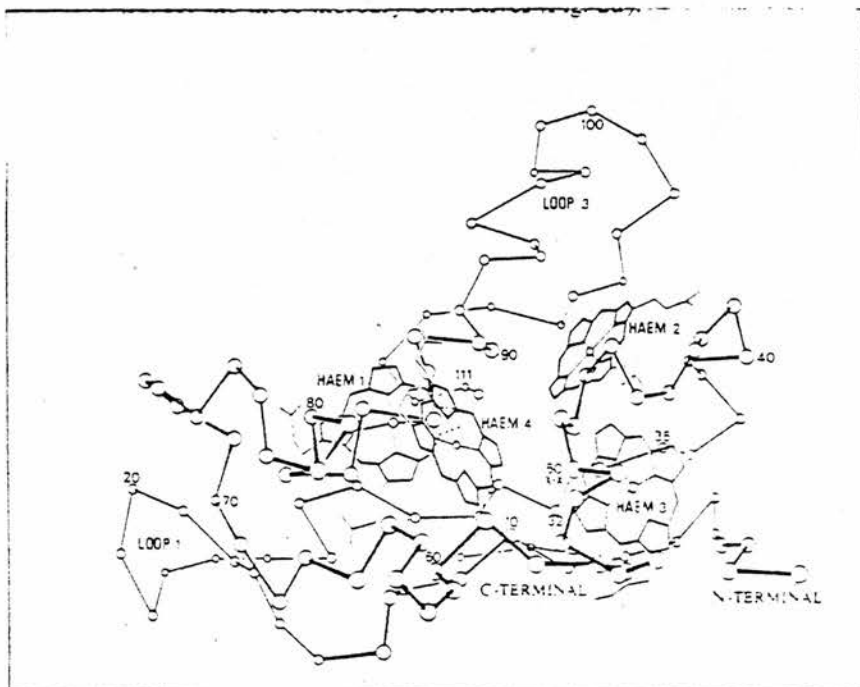
In terms of relevance to this work these two groups need not be discussed any further while the first group will be treated in greater detail. This review is however directed towards giving an overall picture of the properties of the cytochromes c rather than a detailed coverage of the field.

Low spin, high potential cytochromes resembling mitochondrial cytochrome c (with the single haem group attached near the N-terminus of the polypeptide chain and with methionine as the sixth iron ligand) have been isolated from aerobes, facultative anaerobes and photosynthetic bacteria. The best studied are the cytochromes c_2 of photosynthetic bacteria, the mitochondrial cytochromes c, and the cytochromes c_{551} of the Pseudomonads. The proteins consist of a single polypeptide chain of 85 to 135 amino acid residues and possess relatively high midpoint potentials (+150 to +380 mV) (Salemme 1977).

Fig. 2: A. Tertiary Structure Cytochrome c' R. molischianum



B. Tertiary Structure Cytochrome c₃ D. desulfuricans
(Strain Norway)



Legend Fig. 2The Tertiary Structure of the Cytochromes c' and c₃A. Cytochrome c' (R. molischianum)

The molecule is composed of two monomers. The dimer is V-shaped. Each monomer contains four roughly parallel α -helices. The haems are covalently bound near the carboxy termini of the monomers and are separated in space by two helices (A helices), one from each subunit which form the closest interhelical interaction in the molecule. The haem groups are roughly parallel to each other, and are situated at each end of helical bundle with their propionates pointing towards the molecular surface.

B. Cytochrome c₃ (D. desulfuricans strain Norway)

The tertiary structure of the molecule is bean shaped. The α -carbon positions are represented by circles whose size indicates their relative depth. The numbering of the haems is arbitrary. Each haem is distinct in terms of environment and solvent exposure.

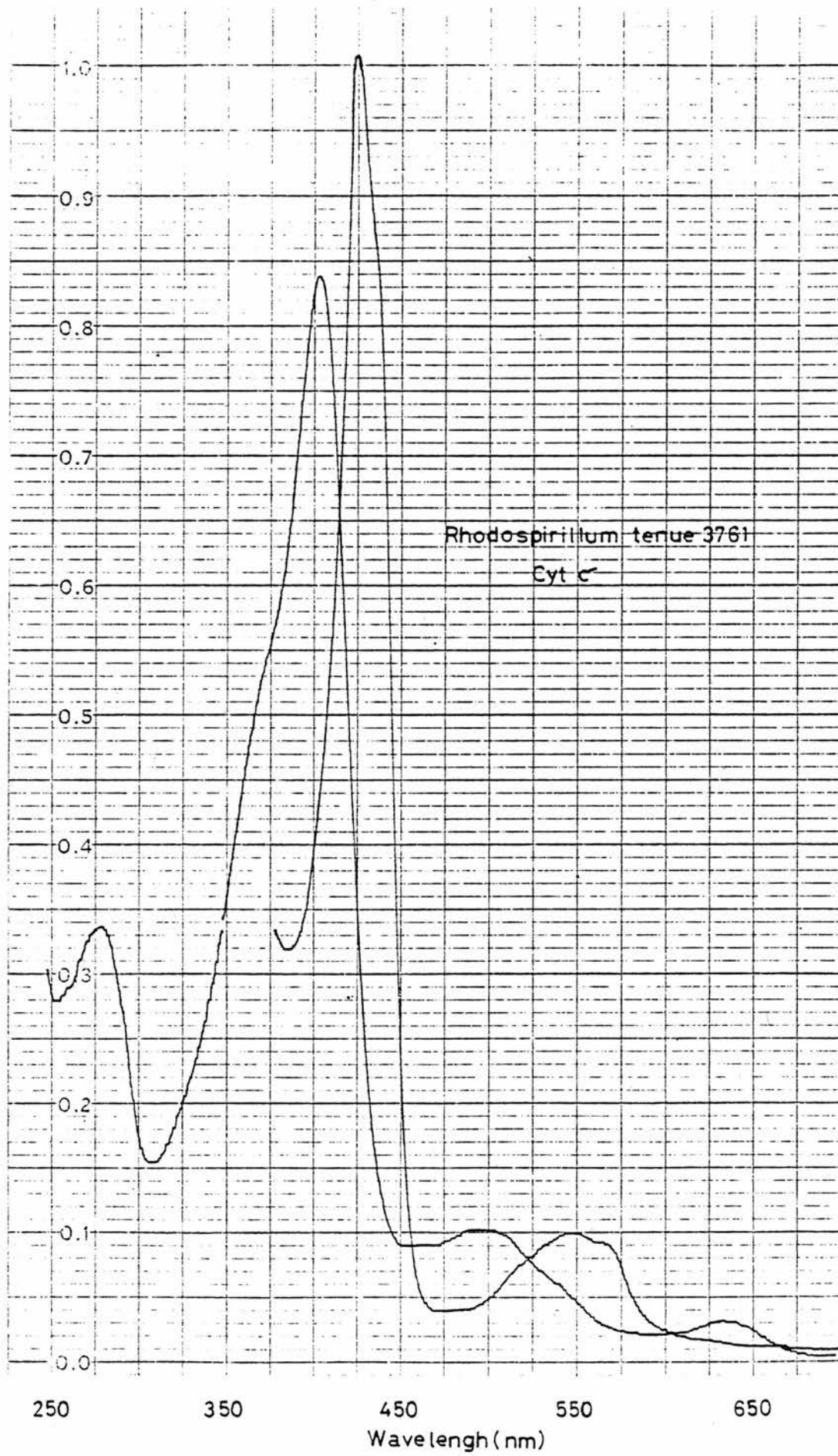
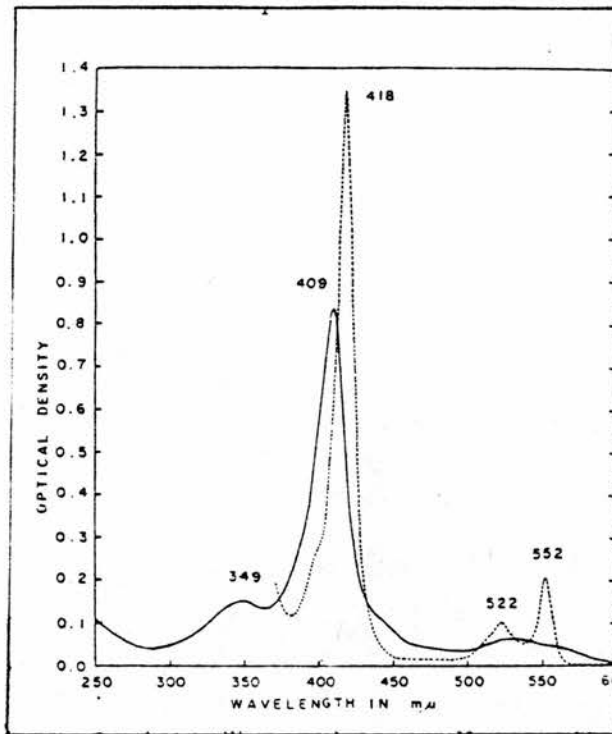
Fig. 3: Absorption Spectrum Cytochrome c' (Rhodospirillum tenue)

Fig. 3: Absorption Spectrum Cytochrome c' (Rhodospirillum tenue)

The absorption spectra of the cytochromes c' are characterized by charge-transfer bands at 490-500 and 630-650 nm, and a broad γ absorption maximum centred at 390-400 nm in the oxidized state. In the reduced form there is a poorly defined absorption maximum at 550-565 nm. The γ band has a maximum near 425 nm and a pronounced shoulder near 430 nm.

Fig. 4: Absorption Spectrum Cytochrome c_3 (*Desulfovibrio desulfuricans*)
(Horio and Kamen 1961)



The cytochromes c_3 spectra are distinguished by the very low absorbance in the ultraviolet region below 300 nm and by the ratio $\epsilon_{\text{red}}/\epsilon_{\text{ox}}$ of approximately 1.5 rather than approximately 1.2 which obtains for most low spin cytochromes c . There is also a characteristic shoulder at the short wavelength side of the reduced Soret peak.

The properties of some of the low spin high potential cytochromes are given in table 1. Other properties will be discussed in relevant sections.

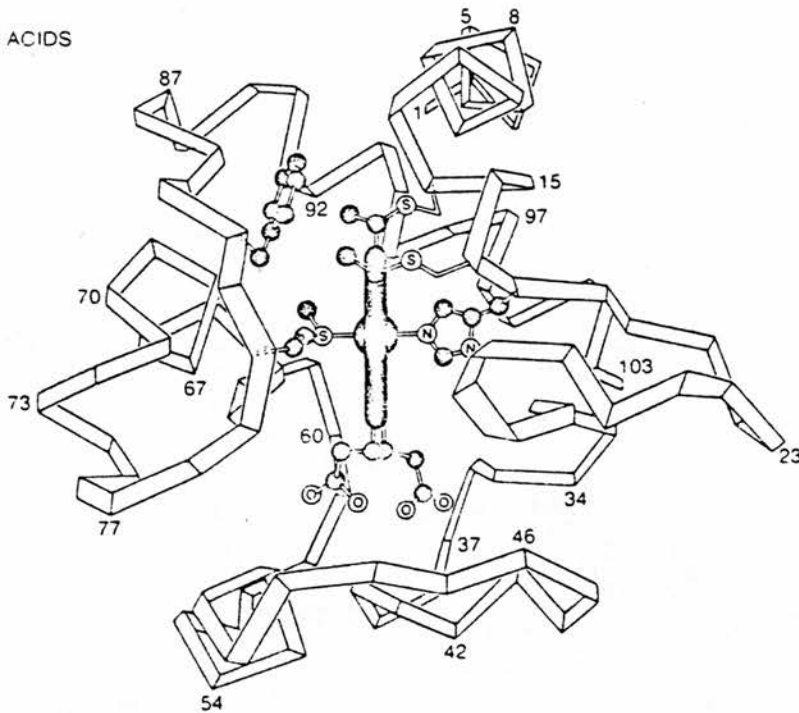
X-ray crystallographic studies indicate that these cytochromes possess a common three dimensional structure (despite variable polypeptide lengths) in which the polypeptide chain is folded around a central haem.

Figs. 5, 6 and 7 give the three dimensional structure ribbon diagrams of cytochromes so far studied. The N-terminal half of the protein forms the right side and bottom of the molecule and provides stabilizing bonds which hold the haem rigid. The left side of the molecule contains several hydrophobic side chains that almost completely shelter the haem and remove it from contact with solvent. The haem propionates do not project into the solvent but are hydrogen bonded to aromatic amino acid residues at the bottom of the molecule. In all these cytochromes - i.e. c, c₂, c₅₅₅, c₅₅₀, and c₅₅₁ there seemed to be need for (i) a tryptophan residue hydrogen bonded to the inner or buried haem propionate; (ii) another nearby aromatic ring; (iii) a serine/threonine hydrogen bond to the outer propionate and (iv) a pair of aromatic side chains to the right of the haem. The differences in size of the different cytochromes is due to extended loops in regions that occur on the surface of the molecule distant from the haem, or the presence of a large loop of residues which forms the bottom of the molecule and shields the two propionic acid residues from solvent. In absence of the latter, downward folding of the left side of the molecule and inward tilting of the bottom of the haem appears to take place as a compensatory mechanism leading to little difference in the extent of haem exposure (Salemme 1977; Almassy and Dickerson 1978; Korszun and Salemme 1977). Korszun and Salemme 1977 offer this as

Fig. 5: The Tertiary Structure of Some Low Spin Cytochromes c

TUNA c
103 AMINO ACIDS

M



RHODOSPIRILLUM c₂
112 AMINO ACIDS

L

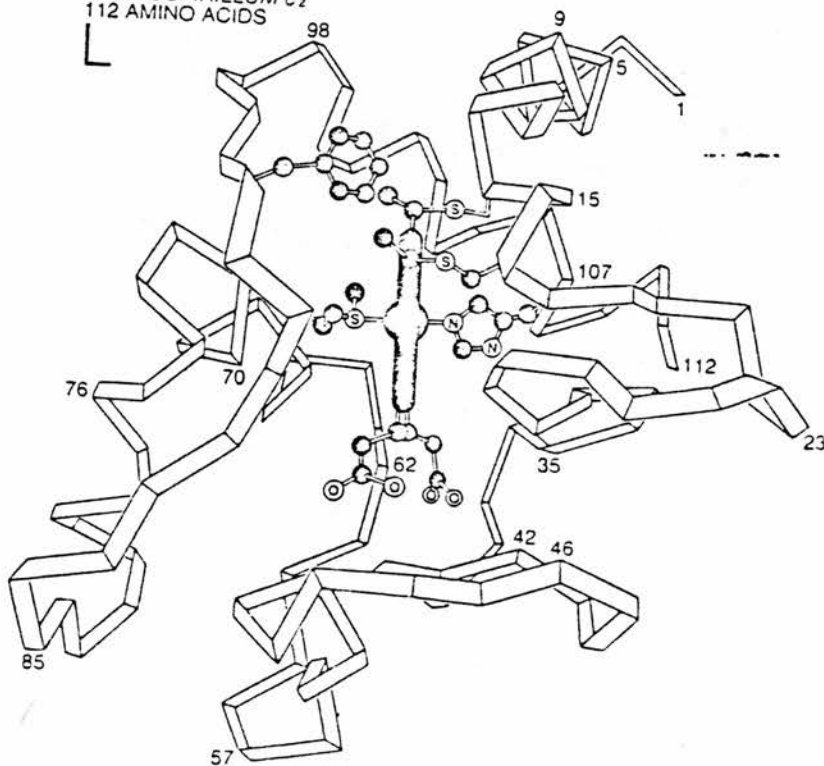


Fig. 6.

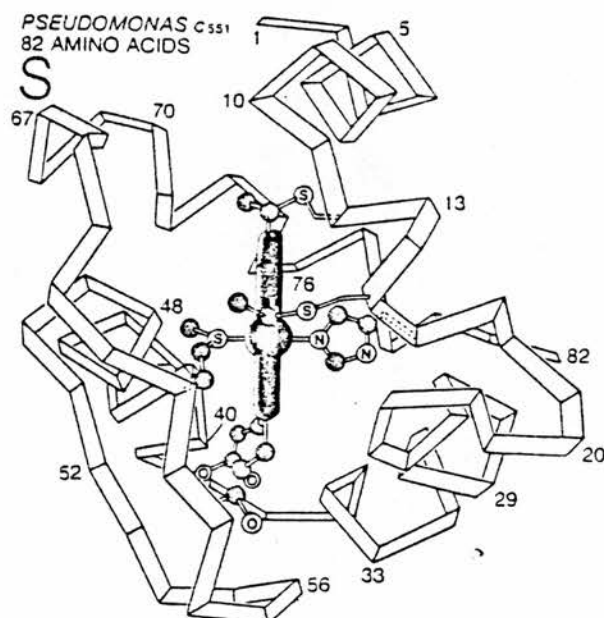
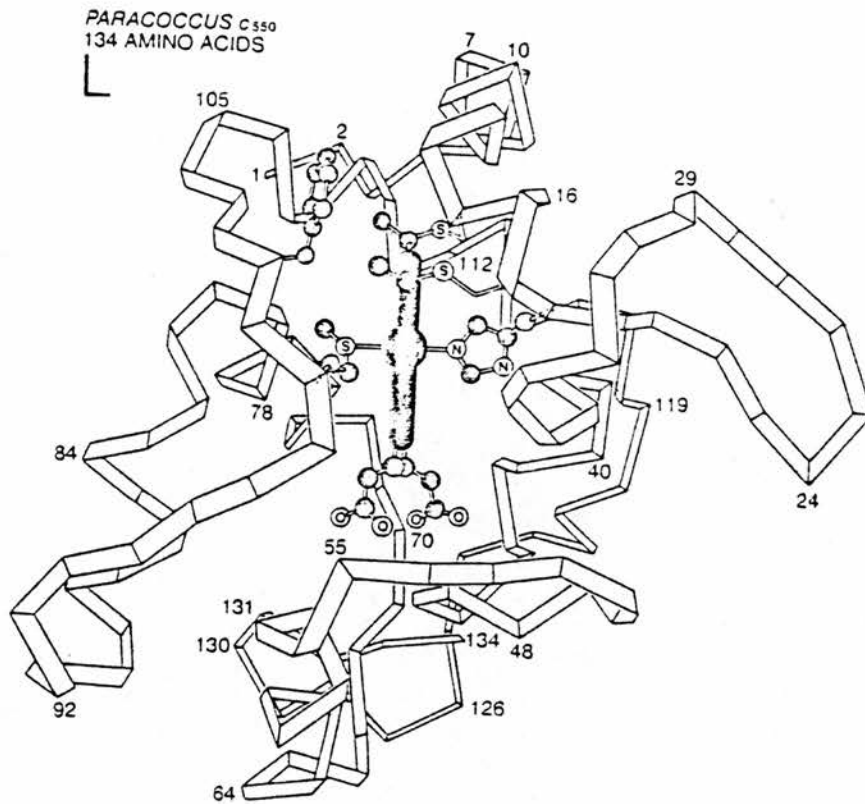
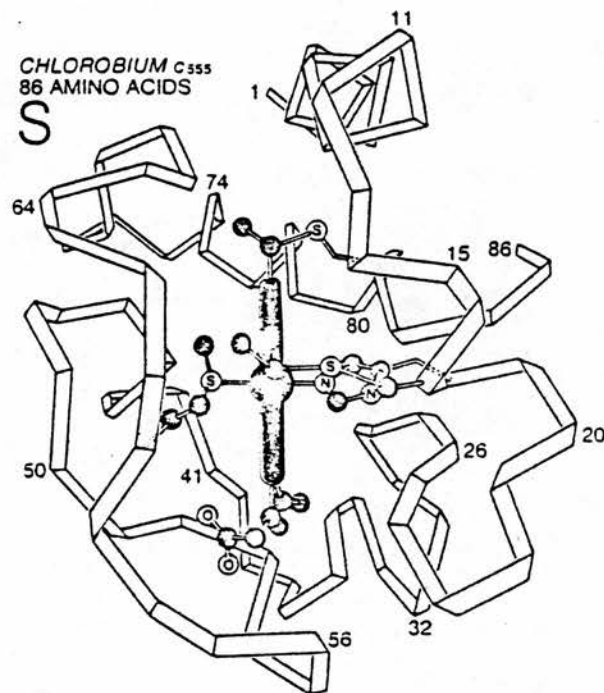


Fig. 7.



Figs. 5, 6 & 7: The Tertiary Structure of Some Low Spin and High
Midpoint Potential Cytochromes c

The protein chain is represented as a folded ribbon. Each fold indicates an alpha carbon atom with its amino acid side chain; numbering is sequentially from the amino terminus of each protein. The flat dark slab is the haem group seen edge on largely buried in the molecule's hydrophobic interior with only one edge exposed at a crevice facing the viewer. The histidine and methionine ligands are to the right and left of the molecule respectively. The propionic acid side chains extend downwards.

Examination of the figures clearly shows the tertiary structure homology and indicates that despite differences in lengths the haem propionates are largely and almost equally shielded from solvent.

evidence against the proposal that the degree of haem exposure due to size differences is a major determinant of redox potentials in cytochromes. Kassner 1972 has proposed that the redox potentials of many high potential cytochromes may be accounted for by a local haem environment of low dielectric constant characteristic of non-polar amino acid side chains and that this factor may play a dominant role in the determination of the oxidation reduction properties of these proteins. NMR spectroscopy coupled with sequence comparison indicate that the immediate environment of the haem is similar in a number of cytochromes c (Cookson et al. 1978).

Despite similarity in tertiary structure low spin cytochromes exhibit individual features. The nature of this treatment of the field excludes detailed enumeration of all low spin cytochromes. Two cytochromes, Pseudomonas c₅₅₁ and Chlorobium c₅₅₅, were chosen for further description. The object is to project similarities of this class of cytochromes while at the same time pointing out individual features which give closely related cytochromes their characteristic properties. It is hoped that such an approach would facilitate comparison with cytochromes described later as well as give an overall picture of the field.

i. Pseudomonas cytochrome c₅₅₁

Pseudomonas aeruginosa cytochrome c₅₅₁ was an important model in producing evidence that haem iron is coordinated to a histidine residue and a methionine residue rather than being a bis histidiny1 adduct. The presence of a single histidine excluded the latter possibility; reaction of bromoacetate with a methionine residue which led to marked changes in properties was a strong indication of the former (Fanger et al. 1967).

The visible absorption spectra of Pseudomonas cytochrome c_{551} are similar to those of the mitochondrial and c_2 cytochromes. The α -peak at 550 nm is symmetrical, the α/β ratio 1.8 and the δ/α ratio 5. The redox potential varies between 270-290 mV. In Pseudomonas aeruginosa c_{551} the redox potential shows pH dependence. This has been interpreted in terms of ionizing groups on the protein with a pKa of 6.2 in the ferricytochrome and 7.3 in the ferrocyclochrome. The pKa 7.3 corresponds to a spectral shift in the α -peak maximum from 551 nm to 553 nm with increasing pH and the α -peak becomes asymmetric. The difference in redox potential between mitochondrial cytochrome c, with a pH independent redox potential, and Pseudomonas aeruginosa c_{551} was interpreted in terms of the haem propionates being more exposed and less hydrogen bonded in the latter than in the former because of tertiary structure differences (Moore et al. 1980). The tertiary structure of Pseudomonas aeruginosa c_{551} has been compared to that of cytochrome c, c_2 and Paracoccus c_{550} (Dickerson, Timkovich and Almasy 1976). Pseudomonas c_{551} lacks a loop of chain at the bottom of the molecule protecting the bottom of the haem crevice relative to mitochondrial cytochrome c (Almasy and Dickerson 1978). This feature is shared by Chlorobium cytochrome c_{555} .

Some evidence for the function of Pseudomonas cytochrome c_{551} is obtained from its reaction with nitrite reductase and cytochrome c_{551} peroxidase. Cytochrome c_{551} was shown to function as an electron donor to both (Horio 1958a and b; Soininen and Ellfolk 1972). The amount of cytochrome c_{551} present in aerobically grown cells of Pseudomonas aeruginosa is considerably less than in those grown anaerobically in the presence of nitrate (Parr et al. 1976). Neither the nitrite reductase nor the peroxidase is specific for cytochrome c_{551} , and the copper protein azurin also

Table 1: Some Properties of Cytochromes of Class I

	Absorption Maxima			Em	M.W.	PI
	α	β	γ	mV	Dalton	
(a) <u>Mitochondrial c</u>	550	521	415	+260	13000	10.5
(b) <u>Cytochrome c₂</u>						
<u>All Rhodospirillaceae</u>						
except <u>R. tenue</u> and	549-	520	416-	+290	11000	5.5
<u>Rps. gelatinosa</u> .	552		419	to	to	to
<u>Paracoccus c₅₅₀</u>				400	14000	9.8
(c) <u>Cytochrome c₅₅₁</u>						
<u>Pseudomonas aeruginosa,</u>						
<u>Pseudomonads, R. tenue,</u>	551	520	416	+286	8100	4.70
<u>Rps. gelatinosa,</u>						
<u>A. vinelandii</u>						
(d) <u>Others</u>						
<u>Petalonia fascia c₅₅₃</u>	553 (549)*	522	416	+360	10500	acidic
<u>Chloropseudomonas</u>						
<u>ethylica c₅₅₅</u>	555 (550)*	523	418	+103	12000	4.65
<u>Chlorobium thiosulphato-</u>	555	523	419	+114	9970	10.50
<u>philum c₅₅₅</u>	(550)*			(pH 8)		

*Indicates shoulder at wavelength.

References:

- (a) Dickerson and Timkovich 1975
- (b) Bartsch 1978
- (c) Dickerson and Timkovich 1975; Ambler et al. 1979a and b;
Van Beeuman 1980
- (d) Yakushiji 1971; Meyer et al. 1968; Shioi et al. 1972

reacts as an electron donor. These observations preclude an essential role for cytochrome c_{551} in denitrification and suggest functional interchangeability with azurin.

ii. Chlorobium Cytochrome c_{555} (and Relatives)

Chlorobium cytochrome c_{555} has been characterized in two species of the green photosynthetic bacteria of the family Chlorobiaceae. The redox potentials are amongst the lowest reported for the low spin high potential cytochromes c being in the range of 100 to 150 mV (Gibson 1961; Shioi et al. 1972; Meyer et al. 1968). The three-dimensional structure of Chlorobium c_{555} is homologous to that of mitochondrial cytochrome c , the cytochromes c_2 and Pseudomonas cytochrome c_{551} (Korzun and Salemm 1977) (fig. 7). The Chlorobium cytochromes c_{555} are monomeric, monohaemproteins. The Chlorobium thiosulfatophilum cytochrome c_{555} - originally reported by Gibson 1961 as c_{554} - is a basic protein of isoelectric point of 10.5 and molecular weight 9970 daltons as determined by amino acid composition and haem content. Its redox potential was found to decrease from 145 mV at pH 6.4 to 114 mV at pH 8.0. The absorption spectrum shows low α/β and α/γ ratios and the α -peak is markedly asymmetric showing similarity to the algal cytochromes f (Meyer et al. 1968). The pH dependence of the redox potential is very similar to that of Pseudomonas aeruginosa cytochrome c_{551} , and has been interpreted in terms of two ionizing groups of pK 6.3 in the ferricytochrome and 7.0 in the ferrocyclochrome. The latter pK corresponds to slight accentuation of the asymmetry of the α -peak (F. Leitch and G.W. Pettigrew - personal communication).

The spectral characteristics of a red-shifted asymmetric α -peak is shared by the algal cytochromes f and a few other cytochromes (Bartsch 1978). Cytochrome $c_{553(550)}$ isolated from Chromatium vinosum has a molecular weight of 13,000 but appears to polymerize in solution.

It has a high potential of 320 mV and is isoelectric at pH 4.4 (Cusanovich and Bartsch 1969). Cytochrome c_{555} of Chloropseudomonas ethylica strain K resembles the algal cytochromes f, not only in spectrum but also in being acidic with an isoelectric point of 4.65, but differs in redox potential (+103 mV) (Shioi et al. 1972). The organism was eventually shown to be a syntrophic mixture of two organisms: the sulphide oxidizing autotrophic green bacterium Prosthecochloris aestuarii and the colourless acetate oxidising sulphur reducing bacterium Desulfuromonas acetoxidans (Gray et al. 1972; Pfennig and Biebel 1976). The function of Chloropseudomonas cytochrome c_{555} is not known; that of Chlorobium c_{555} was studied by Kusai and Yamanaka who isolated a thiosulphate cytochrome c_{551} oxidoreductase from Chlorobium and found that cytochrome c_{555} greatly stimulated the enzymic reduction of cytochrome c_{551} although c_{555} itself was not appreciably reduced in the absence of c_{551} . Mitochondrial cytochrome c could partially substitute for cytochrome c_{555} but several other cytochromes were ineffective. The cytochrome was also found to act as an electron acceptor for a Chlorobium flavocytochrome c-type sulphide dehydrogenase. However yeast cytochrome c was reduced to a greater extent (Kusai and Yamanaka 1973a, b, and c). These observations are consistent with a more specific reaction of cytochrome c_{555} with the thiosulphate reductase than the sulphide dehydrogenase.

B. Bacterial Electron Transport

In contrast to those of higher organisms, which utilize oxygen as terminal electron acceptor, the electron transport chains of bacteria are much more varied with respect to electron carriers and terminal electron acceptors. In addition to oxygen, organic and inorganic compounds (e.g. fumarate, nitrate and sulphate) may function as electron acceptors. Aerobic respiratory systems in bacteria have been reviewed

by Haddock and Jones 1977 and Jones 1977. Detailed information on anaerobic electron transport systems is available for a few compounds only, including fumarate, nitrate and sulphate (Kröger 1977; Konings and Boonstra 1977). The following treatment is an attempt to project the diversity of bacterial electron transport with respect to donor, acceptor and carrier proteins involved. It centres around the best studied organisms and those which appear particularly relevant to the subject of this thesis.

i. Aerobic Hydrogen Metabolism

A multitude of bacteria belonging to different taxonomic groups can utilize hydrogen as an electron donor in the presence of oxygen. These bacteria include members of the genera Alcaligenes, Pseudomonas, Paracoccus, Nocardia and Bacillus. The majority of investigations have been done on bacteria belonging to the genera Pseudomonas (Ishague et al. 1973) and Alcaligenes (Ishague et al. 1970). The organisms vary in their requirement for NAD as an intermediate for electron flow from hydrogen to oxygen.

It has been established that either one or both of two distinct types of hydrogenase may be present depending on the species: a soluble enzyme which will reduce NAD directly with H_2 , and a membrane bound enzyme which does not. The latter donates electrons directly into the respiratory chain, although the soluble enzyme may also initiate electron transport that ultimately reduces O_2 . The soluble hydrogenase generates NADH for CO_2 fixation while the membrane bound form is involved in energy production. Cells grow well autotrophically without the membrane bound enzyme, as the soluble hydrogenase can supply NADH which itself can generate ATP. Mutants lacking the soluble enzyme grow very poorly; NADH can be generated only via energy consuming reversal of electron transport flow at the expense of ATP

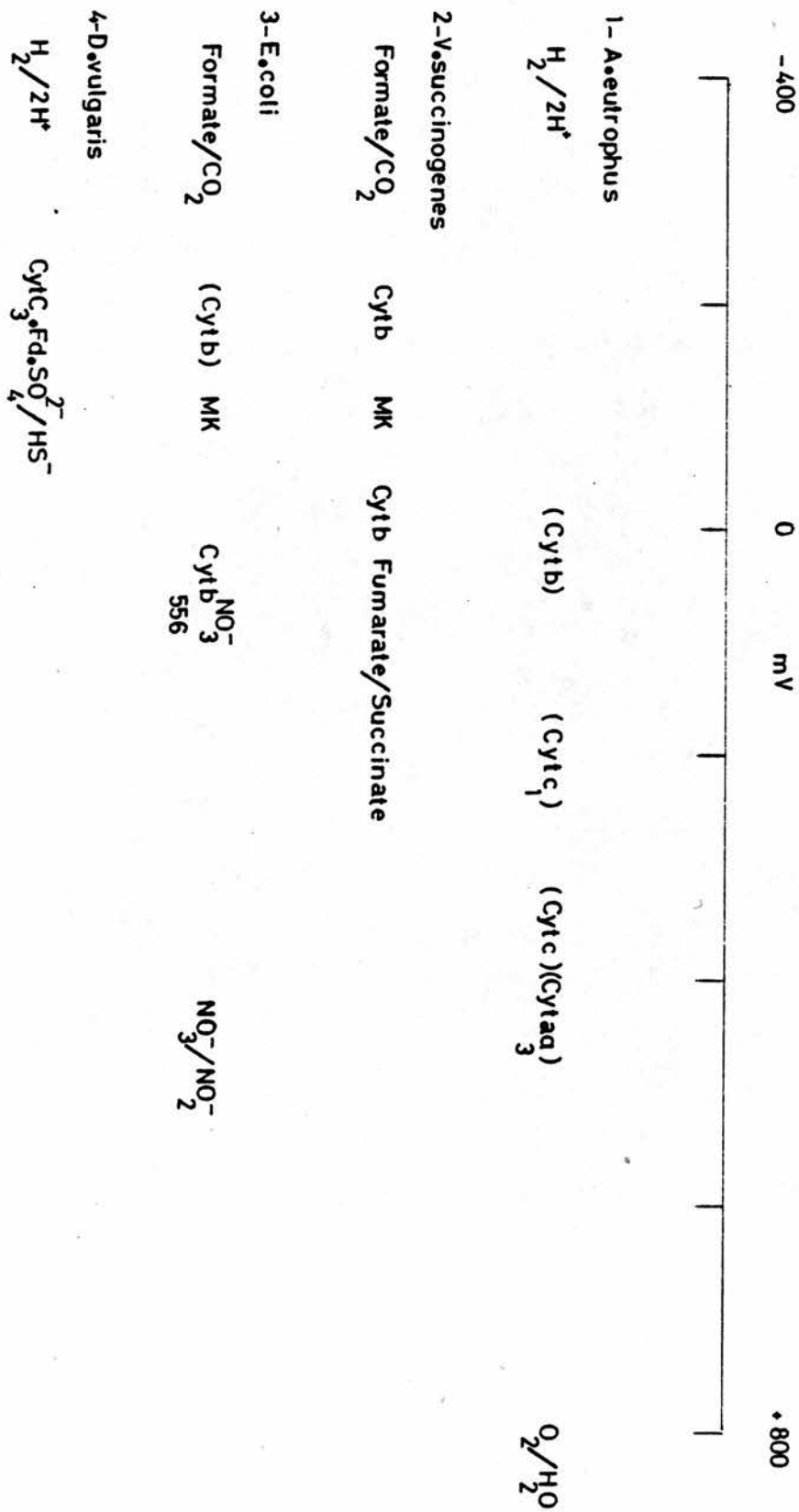
produced via the respiratory chain (Adams et al. 1981). Alcaligenes eutrophus contains both types of hydrogenase while Paracoccus denitrificans contains only the membrane bound one (Schink and Schlegel 1978). All aerobic hydrogen bacteria are facultative autotrophs and are able to grow on $\text{CO}_2 + \text{H}_2$ as well as under heterotrophic conditions on a number of organic compounds as substrates such as sugars, organic acids and amino acids (Schink and Schlegel 1978).

The electron transport chain of A. eutrophus is shown in fig. 8a and is very similar to the mitochondrial chain in organization. It contains cytochromes aa_3 , b_{560} , c_{553} and cytochrome o as shown by difference spectroscopy at room temperature. Difference spectra at low temperatures suggest the presence of extra cytochromes b and c. Membrane fractions could oxidize H_2 , NADH, NADPH as well as formate and succinate. Both formate oxidase and dehydrogenase activities were demonstrated; yet the organism could not grow on formate as the sole electron donor. In the presence of hydrogen, growth on formate occurs indicating that formate replaces CO_2 in autotrophic growth. Moreover CO_2 production from formate by intact cells was observed manometrically. The obvious discrepancy that arises from these observations was not explained (Probst et al. 1976).

ii. Respiration to Fumarate

Fumarate can be synthesized from a variety of amino acids and other metabolites and has a relatively high redox potential (E_o' fumarate/succinate = 33 mV) and it can therefore serve as terminal electron acceptor. Depending on the organism and on growth conditions a number of electron donors can be utilized (Krüger 1978).

The respiratory chain (fig. 8a) consists of at least one membrane bound dehydrogenase that is specific for one of the donors, a naphthoquinone and fumarate reductase. In Vibrio succinogenes a

Fig. 8a: Some Bacterial Electron Transport Systems

cytochrome b ($E_m - 200$ mV) appears to be the acceptor of reducing equivalents from a formate dehydrogenase, as it is reduced by formate in the absence of other redox components. Fumarate reductase contains covalently bound FAD and accepts electrons from another cytochrome b ($E_m - 20$ mV) Kröger et al. 1979. C-type cytochromes identified in V. succinogenes are reported to play no part in this electron transport system. In general it is thought that bacteria which are capable of reducing fumarate contain b type cytochromes; this is thought to be true also for those bacteria which cannot use oxygen as terminal electron acceptor and membrane bound c-type cytochromes are reported as missing in many cases (Kröger 1978). B-type cytochromes linked to fumarate reduction have been reported in D. gigas (Hatchikian and Le Gall 1972), Bacillus megaterium (Kröger and Dadak 1969) and in Mycobacterium phlei (Bogin et al. 1957). In D. gigas an increase in cytochrome b content relative to cytochrome c_3 (and corresponding to an increase in fumarate reductase activity) was observed. The cytochrome b was not detected in a strain with low fumarate reductase activity (Hatchikian and Le Gall 1972). In Propionibacteria cytochrome b was also proposed to be involved in electron transport systems which oxidized NADH, glycerol-1-phosphate and lactate with fumarate as a hydrogen acceptor. In these schemes glycerol, lactate and glucose are converted to oxaloacetate which generates endogenous fumarate. The latter accepts reducing equivalents from reduced nucleotides, being converted to propionate via succinate (De Vries et al. 1973).

E. coli can grow on either glycerol or L-~~α~~-glycerol phosphate as sole source of carbon and energy aerobically but not anaerobically. The addition of nitrate or fumarate to the medium permits anaerobic growth (Kistler and Lin 1972). In cytochromeless mutants it was suggested that fumarate reduction allows glycolysis to proceed by

acting as an electron sink for the reoxidation of NADH. Such mutants cannot form cytochromes unless supplemented with δ -amino laevulinic acid; and can grow anaerobically on glycerol and DL-glycerol-3-phosphate in the absence of δ -amino laevulinic acid with fumarate as the terminal electron acceptor. Cytochrome independent oxidoreductase activities are induced under the growth conditions. Menaquinone was suggested as a component of the oxidoreductase system (Singh and Bragg 1975; 1976).

Utilization of molecular hydrogen as an electron donor to fumarate has been demonstrated for E. coli, Vibrio succinogenes as well as D. gigas (Macy et al. 1976; Jacobs and Wolin 1963; Hatchikian and Le Gall 1972).

The consumption of H_2 provides organisms with a supply of reductant which may be used for the generation of energy. H_2 production on the other hand enables some organisms to dispose of excess reductant in the absence of electron acceptors other than protons. In natural environments the association of H_2 producing and H_2 consuming organisms is sometimes so intimate that the extracellular level of H_2 is extremely low; and a number of organisms have been reported to carry out "interspecies" hydrogen transfer. Such a "consortium" was reported to be formed by Ruminococcus albus in continuous culture with Vibrio succinogenes in the presence of fumarate. The mixed culture pattern was consistent with NADH formed during glycolysis by R. albus being reoxidized by interspecies hydrogen transfer to V. succinogenes which utilizes the gas for reduction of fumarate to succinate. The reaction requires the presence of an NADH-linked hydrogenase in R. albus (Iannotti et al. 1973). This interspecies cooperation allows V. succinogenes to grow at the expense of H_2 produced by R. albus; but it also offers a bonus to the latter in the form of higher ATP gain. In monoculture of R. albus pyruvate is

converted to acetylCoA which is subsequently reduced to ethanol so as to generate NAD from NADH; and ethanol accumulates in the medium. In presence of V. succinogenes, for every acetylCoA shifted from ethanol to acetate formation an extra ATP is generated (Thauer et al. 1977).

iii. Respiration to Sulphate

Sulphate reduction has been demonstrated in members of the genus Desulfovibrio. These are strict anaerobes which require sulphate or thiosulphate as terminal acceptors for respiration, but can also grow by fumarate dismutation and phosphoroclastic cleavage of pyruvate to acetyl phosphate accompanied by hydrogen evolution. The physiology of these organisms has been reviewed by Le Gall and Postgate 1973. Cytochrome c_3 is proposed to be involved in the electron transport system. The thermodynamics of the phosphoroclastic cleavage of pyruvate is interesting. The mid potential of the H^+/H_2 couple (-414 mV) is low as compared to that of cytochrome c_3 (-205 mV). Low partial pressure of H_2 was suggested to render the reaction thermodynamically favourable (Dickerson and Timkovich 1973). The organisms can also grow on H_2 passing the reducing equivalents to oxidized sulphur compounds. Two molecules of ATP are expended in a priming reaction to convert sulphate to adenosine phosphosulphate which is reduced to AMP and sulfite. Not much is known about the subsequent steps in the pathway involving the conversion of sulfite to sulphide. The organisms can survive on hydrogen gas as sole source of reducing equivalents in presence of sulphate as terminal acceptor indicating the generation of more than the 2ATP required for the priming steps and that sulphate reduction does not merely act as an electron sink for reducing equivalents when the organism is grown on lactate or pyruvate. It was noticed that the ferredoxin of D. gigas undergoes an oligomerization leading to different redox potentials. It was hence suggested

that ferredoxin could function at alternate sites in the redox chain allowing either hydrogen evolution or consumption to proceed. Moreover the cytochromes c_3 of D. vulgaris, D. desulfuricans and D. africanus were also reported to exhibit multiple redox potentials (Bianco et al. 1979; Singleton et al. 1979). Xavier et al. 1973 have suggested that the different redox potentials might enable reversible coupling with hydrogenase the enzyme involved in hydrogen utilization and evolution.

Tsuji and Yagi 1980 have studied the growth of D. gigas on lactate and sulphate as energy sources. During the early phases of growth an initial burst of H_2 gas evolution occurs. This has been suggested as a mechanism which allows sufficient substrate level phosphorylation to proceed generating enough ATP for the priming step and conversion of sulphate to sulphite. Once sulphite is produced electron transport proceeds through sulphate reduction. Two different hydrogenases are reported to be involved in this scheme. No explanation was however given to a second "capricious" burst of H_2 gas which indicated that the cells were using protons as electron acceptors in the presence of activated sulphate.

iv. Nitrate Respiration

Nitrate reduction by several electron donors is sufficiently exergonic to allow the formation of 2 mol ATP per mol nitrate, due to the relatively high redox potential of the NO_3^-/NO_2^- couple (+420 mV). The reduction product of nitrate respiration, nitrite, is rather toxic. In a number of organisms nitrite can be further reduced to nitrogen; and in addition to nitrate respiration another electron transfer system in which nitrite reductase functions as a terminal oxidase is involved. In addition to this dissimilatory nitrate reduction, a few organisms can carry out assimilatory nitrate reduction where nitrate is reduced to ammonia which can serve as a source of cell nitrogen.

Organisms reducing nitrate synthesize a specific electron transport chain which includes a nitrate reductase. Electrons are donated to this respiratory chain via primary dehydrogenases which are in general inducible enzymes and the nature of the best electron donor hence depends to a large extent on the growth conditions. The cytochromes involved are usually of the b-type (Könings and Boonstra 1977).

A number of substrates can be utilized to reduce nitrate. NADH, and succinate were the most effective electron donors to nitrate reductase of Paracoccus (micrococcus) denitrificans, followed by lactate, malate and formate (Lam and Nicolas 1969). Whole cells of Thiobacillus denitrificans could reduce nitrite to NO, N_2O , and N_2 when sulphide was the electron donor; where as Na_2SO_3 , $Na_2S_2O_4$, NADH, and NADPH were effective donors to nitrate reductase; and extracts of the cells could catalyze the oxidation of sulphide with either nitrogen, nitrate or nitrite as terminal acceptors (Aminuddin et al. 1973). Klebsiella aerogenes can utilize nitrate as the sole nitrogen source for growth (assimilation) and under anaerobic conditions as an electron acceptor (nitrate respiration). In both processes nitrate is reduced to nitrite through a nitrate reductase. The organism exhibits high levels of cytochrome b content and nitrate reductase activity when grown on nitrate. The cytochrome b was suggested as a carrier in the electron transport system to nitrate, as well as to oxygen under aerobic conditions. Under anaerobic growth conditions in the presence of nitrate a six fold increase in the NADH nitrate reductase activity of the cell membranes was observed as compared to cells grown on NH_4^+ . A similar increase in activity was detected in whole cells when formate was used as an electron donor (Knook et al. 1973).

Nitrate respiration has been most extensively studied in E. coli and this system is the best understood. Formate serves as the most effective electron donor (Ruiz-Herrera and De Moss 1969; Lester and De Moss 1971). In addition to formate, other substrates, such as lactate and L-~~α~~-glycerophosphate, can function as electron donors in cells grown under appropriate conditions. Ruiz Herrera and De Moss 1969 studied the reduction of cytochromes in E. coli and suggested the participation of two distinct cytochromes of the b-type. The growth conditions result in the specific induction of two membrane bound redox carriers cytochrome $b_{556}^{NO_3^-}$ and nitrate reductase whose synthesis is repressed by the presence of oxygen. High levels of formate dehydrogenase are also induced if selenite is present in the medium. O_2 dependent cytochrome oxidation can be demonstrated in the presence of NO_3^- , indicating that aerobic electron transfer is still functional in cells grown under anaerobic conditions in the presence of nitrate. Simultaneous addition of O_2 and NO_3^- to an anaerobic suspension of cells leads to the utilization of oxygen first, followed by NO_3^- (Haddock et al. 1977).

C. The Respiratory Systems of Campylobacters

Interest in members of the genus Campylobacter has been generated by their association with a number of disease conditions in both animals and man. Organisms belonging to the genus are gram negative microaerophilic vibrios. The genus is subdivided into two main groups: catalase positive organisms associated with human and animal disease and a single catalase negative species - sputorum - within which two subspecies sputorum and bubulus were recognized. The genus Campylobacter has been reviewed by Smibert 1978.

Intestinal adenomatosis is a transient condition of the intestinal mucosa which has been reported in a number of animals,

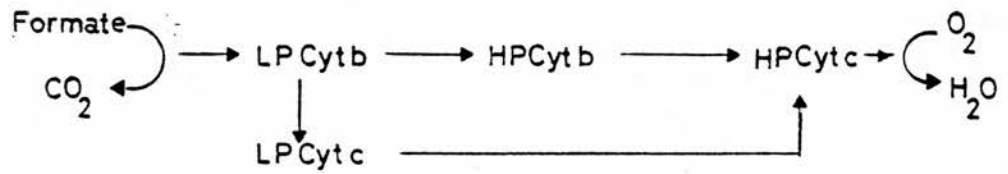
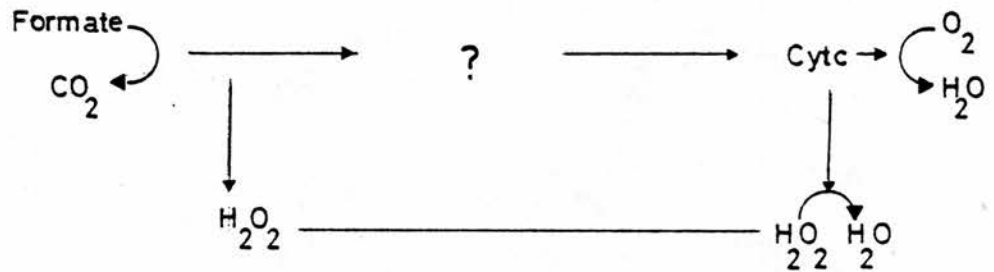
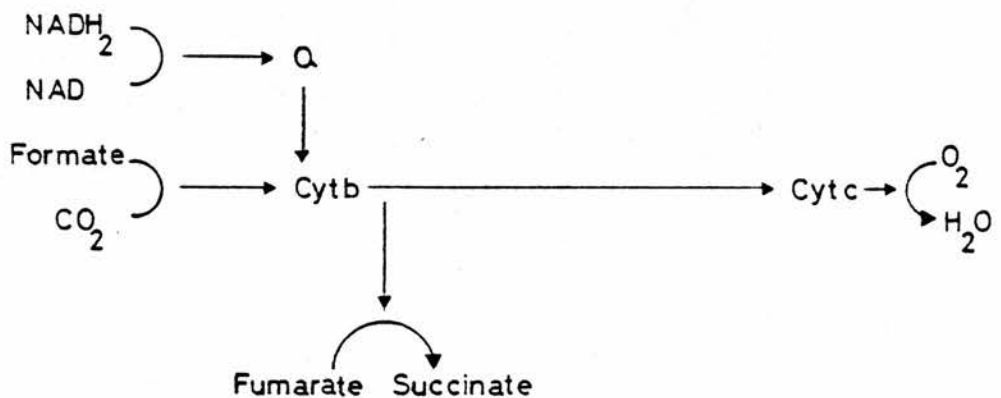
particularly the weaned pig. An organism of the genus Campylobacter was isolated from adenomatous tissue, as well as from pigs with various other intestinal conditions. The adenomatosis vibrio has been compared with representatives of the two subspecies of Campylobacter sputorum from which it could be readily differentiated but with which it shared many features. The subspecies designation "mucosalis" was suggested for the adenomatosis vibrio (Lawson et al. 1975; Rowland et al. 1974; Lawson et al. 1974; Lawson et al. 1976).

The electron transport chains in Campylobacters have been studied in C. fetus subspecies intestinalis (Harvey and Lascelles 1980); in Campylobacter sputorum subspecies bubulus (Niekus et al. 1980a), and in the Campylobacter species of Laanbroek et al. 1978a and b. The organisms can utilize various organic compounds and hydrogen as electron donors, while oxygen acts as an electron acceptor though at low oxygen tensions - i.e. the organisms are microaerophilic. C. sputorum subspecies bubulus and the Campylobacter species of Laanbroek et al. can utilize fumarate and nitrate as electron acceptors under anaerobic conditions (De Vries et al. 1980; Laanbroek et al. 1978a). The latter organism can also utilize a number of inorganic nitrogen and sulphur compounds as terminal acceptors in the presence of hydrogen or formate as electron donors (Laanbroek et al. 1978a and b).

Characterization of these respiratory chains have centred around determination of the path of electron flow through the respiratory chain. The cytochromes present were identified with respect to type and were designated as either of low or high midpoint potentials. This designation was rather qualitative; it was based not on actual potentiometric titrations, but inferred by the reaction of these cytochromes with electron donors such as succinate and ascorbate-TMPD,

or the redox state of these cytochromes in the presence of different electron transport inhibitors. Comparison of the cytochromes of the different Campylobacter species with each other and with cytochromes of the same species grown under different conditions becomes difficult. It may be recalled that an organism such as E. coli has been shown to produce potentiometrically distinct cytochromes b when grown in the presence of different terminal electron acceptors (Reid and Ingledew 1979).

The aerobic electron transport chain proposed for Campylobacter fetus subspecies intestinalis is shown in fig. 8b and proposes the involvement of low and high midpoint potentials cytochromes b and c. The organism cannot grow on formate and fumarate anaerobically despite the presence of high activities of formate dehydrogenase and fumarate reductase in the aerobically grown cells (Harvey and Lascelles 1980). The respiratory scheme in Campylobacter sputorum subspecies bubulus (fig. 8b) proposes electron transport from formate through unidentified intermediates via cytochrome c to oxygen. The presence of a formate oxidase with low affinity for oxygen leads to the production of H_2O_2 which is disposed of by a cytochrome c peroxidase. The microaerophilic nature of the organism was explained in terms of inactivation of formate dehydrogenase by H_2O_2 at high oxygen tension (Niekus et al. 1980b). The organism shows similarity to Vibrio succinogenes in this respect (Jacobs and Wolin 1963). The function of cytochrome b, spectrally identified in Campylobacter sputorum subspecies bubulus, was not defined (Niekus et al. 1980a). The involvement of cytochrome b in fumarate reduction was demonstrated in Campylobacter species of Laanbroek et al. 1978 (fig. 8b). Cytochrome c identified under the same growth conditions was proposed to function in oxygen respiration. Similar observations were made in Vibrio succinogenes (Jacobs and

Fig. 8b: The Respiratory Chains of Some Campylobactersa-*C.fetus* ss *intestinalis*b-*C.sputorum* ss *bubulus*c-*Campylobacter* sp. DSM 806

LP = Low potential

HP = High potential

Q = Menaquinone

Harvey and Lascelles 1980

Niekus et al. 1930a

Laanbroek et al. 1978a & b

Wolin 1963; Krüger and Innerhoffer 1976). Campylobacter species could also utilize H_2 from another organism in mixed culture - interspecies hydrogen transfer - again showing similarity to Vibrio succinogenes.

The similarities between these closely related organisms can be summarized in the following: (i) their microaerophilic nature; (ii) the ability to utilize formate or hydrogen as electron donors; (iii) with the exception of Campylobacter fetus subspecies intestinalis, the ability to grow on fumarate and nitrate anaerobically; (iv) the presence of both b-type and c-type cytochromes of 'low' and 'high' midpoint potentials.

The least characterized electron transport chain is that of Campylobacter sputorum subspecies mucosalis. The organism can grow microaerophilically on blood agar plates under an atmosphere of hydrogen, showing similarity to Vibrio succinogenes, but indicating requirement for an essential growth factor. The organism contains a functional electron transport chain as evidenced by cytochrome c reduction by formate and oxidation of the reduced cytochrome by O_2 . Anaerobic growth on formate/succinate on blood agar plates and reduction of nitrate and nitrite were also demonstrated (Lawson et al. 1975; Lawson et al. 1981). The organism can grow and divide within parasitized cells. The electron donor(s) under such conditions are not known; the acceptor is presumably oxygen.

An important step towards understanding the metabolic relationship of Campylobacter sputorum subspecies mucosalis to its host cell is the study of the metabolism and mode of energy generation of the organism "in vitro" under different growth conditions.

A number of approaches are available in studying electron transport chains:

- a) the determination of the type of cytochromes in whole cells, extracts or membrane fractions spectrally in the presence or absence of ligands such as CO or alkaline pyridine which produce characteristic modifications of the spectrum;
- b) the study of the redox state of the individual components of an electron transport chain as a function of the addition of different reductants and oxidants;
- c) the effect of selective inhibitors on the redox state of different components;
- d) measurement of the redox potentials of the different components;
- e) isolation and purification of the components and the study of their reactivity in reconstitution experiments.

The present study proposes to identify the C-type cytochromes of Campylobacter sputorum subspecies mucosalis potentiometrically and to isolate and characterize these cytochromes.

CHAPTER II

METHODS

A. The Growth and Harvesting of Campylobacter Sputorum Subspecies Mucosalis

i. Growth Conditions

Seed organisms of Campylobacter sputorum subspecies mucosalis strain FS253/72 (NCTC 11000) were kindly supplied by Dr G H K Lawson of the Department of Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh.

The organisms were grown on blood agar plates under microaerophilic conditions in an atmosphere of 4% O₂, 5% CO₂, 16% N₂ and 75% H₂ for 48 hours at 37°C (Lawson et al. 1974).

The cells were grown on the blood agar so that they formed distinct streaks which made visual detection of contaminants forming discrete colonies an easy task. Nevertheless the cells were examined at intervals by gram staining and by reaction with antiserum to ensure that the original strain was maintained throughout subculturing.

ii. Gram Stain

The organisms on a platinum loop were smeared together with a drop of water on a glass slide. The slide was then gently dried by passing over a flame and stained in the following manner:

- a) the slide was flooded with methyl violet solution and left for 2 minutes;
- b) methyl violet was washed off with distilled water and the slide flooded with Gram's iodine solution and left for one minute;
- c) the slide was decolourized by washing with acetone for 3 seconds; washed with water and counter-stained with dilute Carbol Fuchsin for one minute; washed with water, dried gently over the flame and examined microscopically under the oil immersion lens. The organisms which are gram negative appear as pinkish vibroid cells.

iii. Reaction with Antiserum

Antiserum was kindly provided by Dr G H K Lawson. A loop of organisms was mixed into a drop of water on a glass slide. A drop of antiserum was then added, whereupon rapid agglutination could be observed.

iv. Harvesting of Cells

The organisms were harvested by scraping the cells from the surface of each blood agar plate with a sterile glass slide into 1 ml 5 mM sterile sodium phosphate buffer pH 7.0. The resulting cell suspension was then pipetted off into a sterile universal bottle. The cell suspension was centrifuged at 3000 rpm (1000 g) in an MSE Model 2L centrifuge for 30 minutes at 4°C. The supernatant was removed and the cells washed with sterile phosphate buffer to remove traces of haemoglobin and centrifuged again at the same speed. The supernatant was then removed and 1 ml sterile phosphate buffer layered over the cells which were then gassed with argon and kept at -40°C till used.

B. Extraction of Campylobacter Cytochromes c

Bacterial proteins can be extracted by a variety of methods including freezing and thawing, high ionic strength extraction and the use of ionic and nonionic detergents. Combinations of these methods were tested with the objective of maximizing yield. The final procedure adopted for routine extraction involved freezing and thawing, the use of relatively high ionic strength, manual homogenization and passage of cells through a French press. The procedure is depicted in the flow diagram(fig. 9). 59% of the total cytochrome present in the cells was recovered in the extract. When such an extract was centrifuged at 50,000 rpm (165,000 g) for 15 hours, approximately 70% of the extracted cytochrome was recovered as a red glassy pellet.

Fig. 9.

Extraction Procedure

1 gram (wet weight) cells frozen
and thawed twice

+

3-5 ml 25 mM sodium phosphate buffer
pH 7.0/100 mM NaCl



Manual homogenization in a glass
homogenizer at 4°C, freezing and thawing



Passage through French press*
at 12,000 PSI



Centrifugation at 15,000 rpm
(20,000 g) for 30 minutes
at 4°C (Beckman J21 centrifuge)

*Aminco, Silversprings, Maryland, U.S.A.

C. Determination of Cytochrome c Content

Measurement of the absorption maxima at either 410 nm in the oxidized state or at the α -peak in the reduced state, or the alkaline pyridine haemochrome method, were used for quantifying cytochrome c in crude extracts and chromatographic fractions. In the first two methods $\epsilon_{\text{mM}}_{410 \text{ nm}} = 100$ and $\epsilon_{\text{mM}}_{550} (\text{red-ox}) = 20$ were assumed. Such values are approximations characteristic of c-type cytochromes.

The pyridine haemochrome method is however the most accurate of the three since, in the presence of alkaline pyridine, the absorption at the α -peak is solely due to the haem moiety and is not affected by the protein environment. Pyridine and NaOH, to final concentrations of 2M and 0.2M respectively, were added to the cytochrome and the absorption spectrum between 510 and 570 nm was then recorded. A few grains of sodium dithionite were then added, and the reduced spectrum over the same wavelength range was also recorded. The cytochrome concentration was calculated from $\epsilon_{\text{mM}}_{550} = 31.18$ for the reduced spectrum and $\epsilon_{\text{mM}}_{550} = 19.1$ for the reduced minus oxidized spectrum (Bartsch 1971).

D. Redox Potentiometry

i. Theoretical Aspects

Theoretical and technical aspects of redox potentiometry have been reviewed by Dutton and Wilson 1974, Wilson 1978 and Dutton 1978. A redox couple has the tendency to donate or accept electrons according to its electrode potential under specified conditions. The electrode potential is given by the Nernst equation which defines the relationship between the observed potential and the concentrations of the oxidized and reduced species of that particular couple:

$$E = E_m + \frac{RT}{nF} \ln \frac{(\text{ox})}{(\text{red})}$$

where: E is the observed potential in volts in relation to the Normal Hydrogen Electrode (NHE) or any other standard electrode calibrated with reference to the Normal Hydrogen Electrode.

E_m is the midpoint potential in volts of the system when $(ox) = (red)$ and $\ln (ox)/(red) = 0$. In the biochemical field this value is often determined at pH 7.0 (E_{m7}).

(ox) and (red) refer to the activities of the species, though usually molar concentrations or other parameters proportional to these e.g. light absorption are used.

R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$)

T is the absolute temperature in $^{\circ}$ Kelvin

F is the Faraday constant ($96487 \text{ J V}^{-1} \text{ mol}^{-1}$)

n is the number of electrons transferred during the redox reaction.

For biochemical systems at 30°C and pH 7.0 the above relationship simplifies to:

$$E = E_m + \frac{0.06}{n} \log \frac{(ox)}{(red)}$$

ii. General Experimental Procedure

Redox potentiometry was carried out on the total cell extract, and the 100,000 g supernatant and sedimentable fraction. An anaerobic cuvette was set up inside a spectrophotometer (Unicam SP 1800) in such a manner that the potential and the state of reduction of the cytochromes at 510-570 nm could be simultaneously recorded.

$2.5 \times 10^{-3} - 10 \times 10^{-3} \text{ mM}$ final concentration of the cytochromes was enough to give a spectrum suitable for analysis on the 0-0.2 scale of the spectrophotometer. This corresponds to 10-40 nmol of cytochrome in a final volume of 4 ml.

The anaerobic cuvette included a platinum Ag/AgCl combination electrode (Russel Instruments) which projected through a rubber bung into the reaction mixture (fig. 10). This electrode was connected to a pH meter (Corning Eel 110) set to read absolute millivolts. Two syringe needles inserted through the rubber bung acted as inlets and outlets for argon gas. Through a third needle small aliquots of titrant could be added from a Hamilton syringe with a thin long needle directly into the titration mixture, which was continuously stirred magnetically.

Argon gas, saturated with water vapour, was continuously passed over the surface of the solution or bubbled through it, while all titrants were continuously bubbled with the gas.

After the solution had been rendered anaerobic the titration was performed by μ l additions of one of 3 titrants, namely: 50 mM sodium formate (HCOOH/CO_2 $E'_0 = -420$ mV); 100 mM sodium ascorbate (ascorbate/dehydroascorbate $E'_0 = +58$ mV); or 150 mM NADH (NADH/NAD^+ $E'_0 = -320$ mV). In the latter case 0.5 mg commercial NADH/cytochrome c reductase was added to the cuvette contents. One or more of the redox mediators given in table 2 were used during titrations.

iii. Calibration of the Redox Electrode

The redox couple $\text{Fe}^{2+}/\text{Fe}^{3+}$ EDTA was chosen for calibration of the Ag/AgCl electrode because its redox behaviour is well characterized and the oxidized and reduced states of the couple can be accurately made up and are reasonably stable. The midpoint potential of this couple is independent of pH between pH 4 and 6. Above pH 7 this midpoint potential is also independent of the ratio EDTA/total Fe provided this ratio is greater than one (Shwarzenbach and Heller 1951; Kolthoff and Auerbach 1952).

Fig. 10.

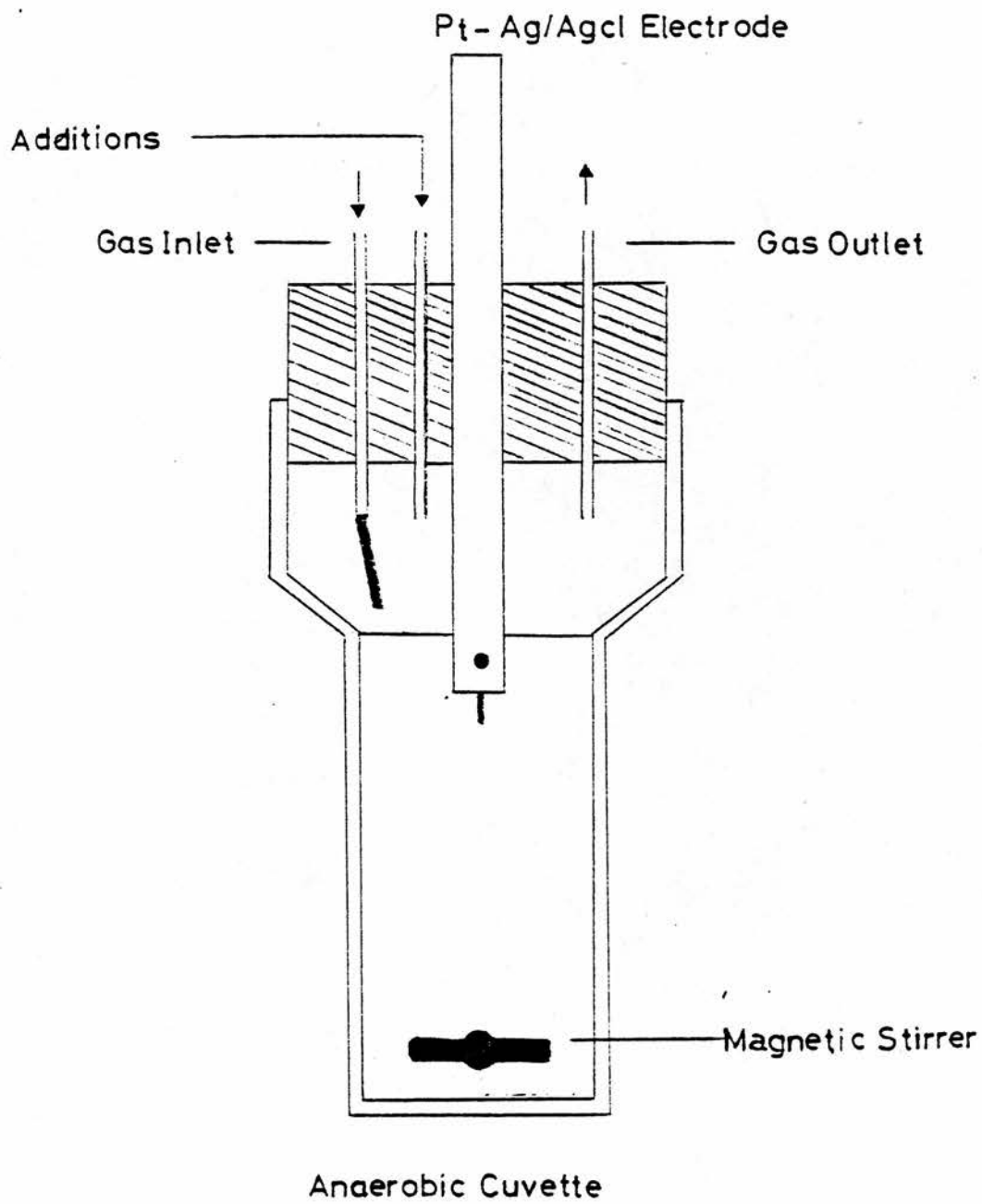


Table 2: Mediators used during Titration of Campylobacter
Sputorum subspecies mucosalis cytochromes
(Wilson 1978)

<u>Mediator</u>	<u>Em mV</u>	<u>n</u>
N,N,N',N' tetramethyl-P-phenylene diamine (TMPD)	+260	1
Diaminodurool (DAD)	+220	2
Fe ³⁺ EDTA	+96	1
Phenazinemethosulphate (PMS)	+80	2
Phenazine-ethosulphate (PES)	+55	2
Duroquinone (DQ)	+5	2
2-Hydroxy-1,4-naphthoquinone (HNQ)	-145	2
Flavin mononucleotide (FMN)	-219	2
Benzyl Viologen (BV)	-311	1

Kolthoff and Auerbach give a midpoint potential of 112 mV for the $\text{Fe}^{2+}/\text{Fe}^{3+}$ couple at pH 5.0.

Reagents:

- a) 2 mM ferric ammonium sulphate/40 mM EDTA.
- b) 100 mM ferrous ammonium sulphate in H_2O which has been equilibrated with argon.

Both solutions a) and b) were kept in dark bottles and used within 12 hours of preparation.

- c) 1.0M sodium acetate buffer pH 4.5.

Electrode Calibration

To the anaerobic cuvette solutions (a) and (c) were added to produce final concentrations of 0.5 mM Fe^{3+} /10 mM EDTA, and 0.1M sodium acetate pH 4.5 in a final volume of 5 ml. The mixture was rendered anaerobic by bubbling argon through it for 15 minutes prior to additions of Fe^{2+} . The bubbling was continued throughout titration. Recorded potentials were then plotted vs $\log \frac{(\text{Fe}^{3+})}{(\text{Fe}^{2+})}$. The potential of the Ag/AgCl electrode was calculated from the equation:

$$\begin{array}{ccc} E_{\text{obs}} (\text{Fe Couple}) & E (\text{Fe Couple}) & \text{S.E.P. Ag/AgCl} \\ & = & - \\ (\text{vs Ag/AgCl electrode}) & (\text{vs N.H.E.}) & (\text{vs N.H.E.}) \end{array}$$

where:

E_{obs} : the observed midpoint potential of the Fe EDTA couple determined from the plotted graph at:

$$\log \frac{(\text{Fe}^{3+})}{(\text{Fe}^{2+})} = 0$$

E_m (Fe Couple) : the midpoint potential of the Fe couple (112 mV)
 vs N.H.E. versus the Normal Hydrogen Electrode (N.H.E.)
 S.E.P. Ag/AgCl : the standard electrode potential of the Ag/AgCl
 vs N.H.E. electrode versus the Normal Hydrogen Electrode

$$\therefore \text{S.E.P.}_{\text{Ag/AgCl}} = 112 - E_{m_{\text{obs}}}$$

iv. Mathematical Treatment of Data: The resolution of multi-component redox titration curves.

(a) Theory

In a redox titration involving one electron transfer the Nernst equation relates the observed potential E (volts) to the relative amounts of oxidized and reduced species in the following manner:

$$E = E_m + 0.06 \log_{10} \frac{(\text{ox})}{(\text{red})} \text{ at } 30^\circ\text{C} \quad \dots\dots(1)$$

alternatively:

$$\begin{aligned} E &= E_m + 0.06 \log_{10} \frac{(T_o - R_o)}{R_o} \\ &= E_m + 0.06 \log_{10} \left(\frac{T_o}{R_o} - 1 \right) \end{aligned}$$

and in general:

$$T_o = R_o (1 + 10^{(E-E_m)/0.06}) \quad \dots\dots(2)$$

where:

(ox) : denotes molar concentration of the oxidized species

(red): denotes molar concentration of the reduced species

T_o : is the total amount of reducible material

R_o : is the amount reduced at any particular point

E : is the observed potential.

For a one component system plotting E vs $\log_{10} \frac{(\text{ox})}{(\text{red})}$ gives a linear plot with a 60 mV slope. E_m , the midpoint potential, is then equal to E when $\log_{10} \frac{(\text{ox})}{(\text{red})} = 0$;

$$\text{i.e. when } \frac{(\text{ox})}{(\text{red})} = 1.$$

Redox titrations of many biological electron transfer systems involve more than one redox component. For such systems the Nernst plot becomes sigmoidal, or even more complex for three or more component systems, approaching linearity only at the extremities.

The Nernst equation is a linear equation of the form $y = mx + c$. The complex curves observed for systems involving two or more components are due to the fact that no consideration is taken of the fact that $T_o = T_1 + T_2 + T_3 + \dots$ etc. when plotting E vs $\log \left(\frac{T_o}{R_o} - 1 \right)$ where T_1 is the total reducible amount of one component, T_2 of the second etc., and T_o is the total reducible material observed on full reduction of all components. It should thus be possible to resolve the system into its constituent components provided T_1 , T_2 and T_3 etc. could be determined and the respective Nernst plots carried out.

To illustrate the approaches used in resolution of multi-cytochrome system during this work a theoretical curve, fig. 11, has been constructed for two components with the following chosen parameters.

First component

$$E_{m_1} = +50 \text{ mV}$$

$$T_1 = 9 \text{ units}$$

Second component

$$E_{m_2} = -100 \text{ mV}$$

$$T_2 = 25 \text{ units}$$

$$T_o = T_1 + T_2 = 34 \text{ units.}$$

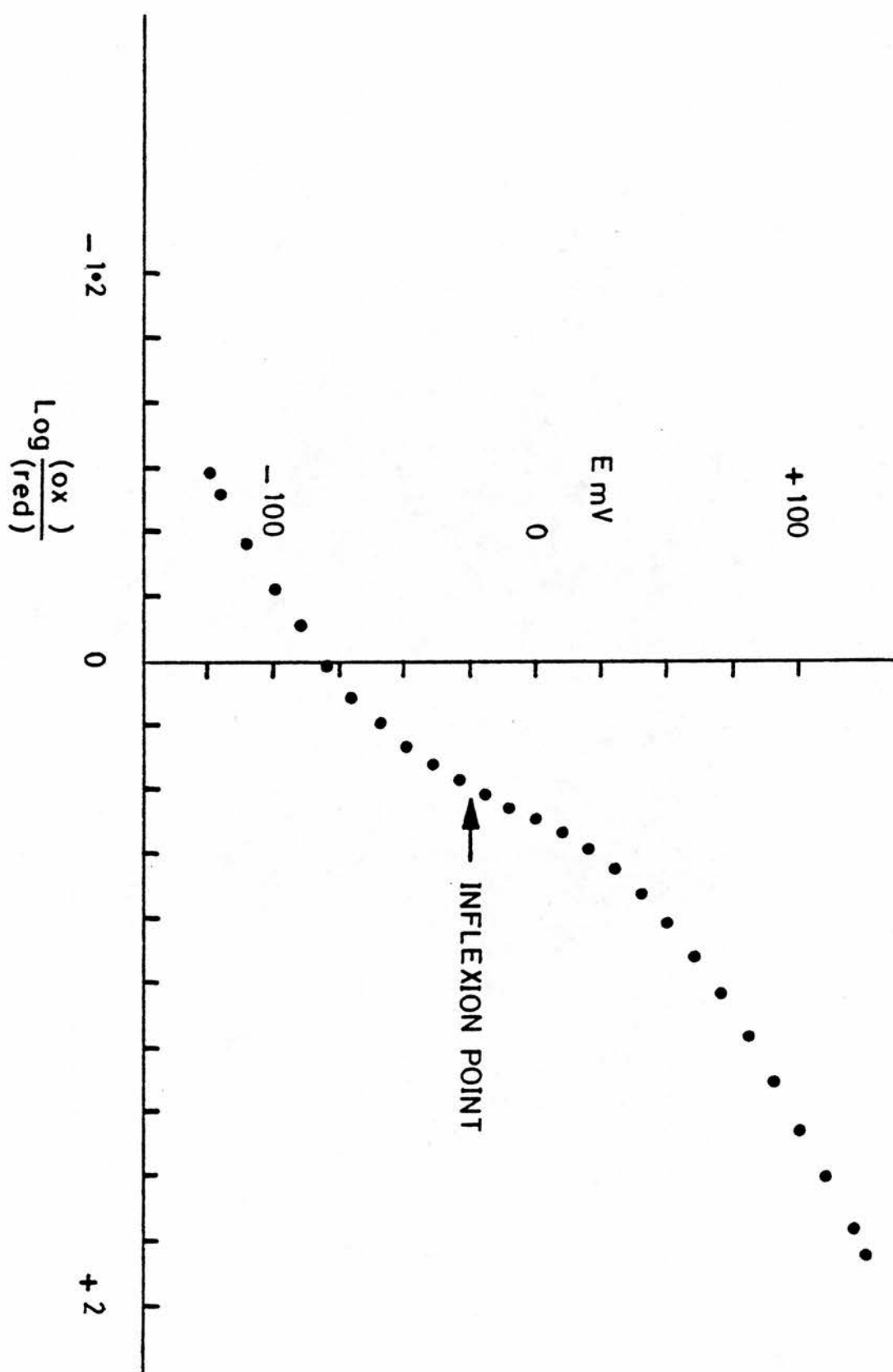
Fig. 11: Theoretical Redox Titration Curve of a Two Component System

Fig. 11: Theoretical Redox Titration Curve of a Two Component System

The curve represents two components of E_m +50 mV and -100 mV and a contribution of 9 units and 25 units to the total reducible material. The inflexion point directly gives the relative contribution of the two components (text). Other methods for resolution of the curve are discussed in text.

To construct such a curve the amount of oxidized and reduced species of each component are calculated at chosen potentials E_1, E_2, E_3 etc. The oxidized species at each potential are then added and so are the reduced species; and $\log_{10} \left(\frac{ox_a + ox_b}{red_a + red_b} \right)$ plotted vs the potentials; where ox_a refers to the oxidized species of the first component, ox_b that of the second component and red_a and red_b are the corresponding reduced species.

In the subsequent treatment matters are reversed and it is assumed that the following parameters are known as experimentally determined values:

$$T_o = 34 \text{ units}$$

E_1, E_2, E_3 etc. are now determined potentials

Ro_1, Ro_2, Ro_3 : the amount of material reduced at each potential

$$\text{i.e. } Ro_1 = (red_{a_1} + red_{b_1})$$

$$\text{and } (ox_{a_1} + ox_{b_1}) = T_o - Ro_1.$$

(b) Resolution of the Curve into its Components

The problem is how to use such experimental data (as depicted by the titration curve) to determine the relative amounts of the constituent components and their midpoint potentials. Three methods (or combinations of these methods) have been used for such analysis.

The methods were used mainly to obtain values for T_1 and T_2 the contributions of each component to the total reducible material. Once T_1 and T_2 were known, they could be used to calculate $\log \left(\frac{ox}{red} \right)$ for each component at the observed potentials. In such calculations however it was assumed that the amount of reduction at the observed potentials correspond to the reduction of one component only and that the reduction of the second component is so small as not to

significantly interfere with the calculations e.g. for a point within the domain of the first component:

$$R_{o1} = \text{red}_{a1} \text{ rather than}$$

$$R_{o1} = (\text{red}_{a1} + \text{red}_{b1})$$

i.e. the contribution of the second component is assumed to be negligible and $\left(\frac{\text{ox}}{\text{red}}\right) = \left(\frac{T_1 - R_{o1}}{T_1}\right)$ and so on for all points falling within the amount of reduction corresponding to the first component.

The assumption made above is generally valid if the midpoint potentials of the two components are 100 mV or more apart.

The same assumption was made for points falling within the second component. The observed potentials were then plotted vs the corresponding values of $\log \left(\frac{\text{ox}}{\text{red}}\right)$ for each component. The midpoint potentials were obtained from a theoretical 60 mV slope line drawn through the points so as to give the best fit. The midpoint potentials were then found from the y axis at $\log \left(\frac{\text{ox}}{\text{red}}\right) = 0$.

Method 1: From the inflexion point(s) (Dutton and Wilson 1974)

In a system containing two components whose E_m values differ by 100 mV or more, the amount of reduced and oxidized material at the inflexion point represent the contributions of the two components.

Reference to the constructed curve (fig. 11) shows that an inflexion point corresponding to $\log \left(\frac{\text{ox}}{\text{red}}\right) = 0.40$ occurs on the curve:

$$\therefore \log \left(\frac{\text{ox}}{\text{red}}\right) = 0.40$$

$$\text{and } \frac{(\text{ox})}{(\text{red})} = 2.51$$

$$\begin{aligned} \text{and } (\text{red}) &= \frac{1}{2.51 + 1} \times T_o \\ &= \frac{1}{3.51} \times 34 \end{aligned}$$

hence at the inflexion point:

$$(\text{red}) = T_1 = 9.68 \text{ units}$$

$$(\text{ox}) = T_2 = 24.32 \text{ units.}$$

The calculated values are not far removed from the theoretical values and can be used to calculate $\log \left(\frac{\text{ox}}{\text{red}} \right)$ values for each component.

However, in experimental curves inflexion points were not always clearly seen and other methods for curve resolution were used.

Method 2: From simultaneous equations

Again the assumption was made that, at linear parts of the curve,

$$R_o = \text{red}_a \text{ rather than}$$

$$R_o = (\text{red}_a + \text{red}_b).$$

Points were then taken which are not at the middle of the curve i.e. on the linear portion of the curve and on the same side e.g. in this case where:

$$E = 120 \text{ mV and } R_o = 0.58$$

$$E = 70 \text{ mV } R_o = 2.9$$

Applying the relationship:

$$E = E_m + 0.06 \log \left(\frac{T-R}{R} \right)$$

it can be found by simultaneous equations that:

$$T = 9.36 \text{ and } E_m = 49 \text{ mV.}$$

These figures are not far removed from the theoretical figures for the first component ($T = 9.0$ and $E_m = 50 \text{ mV}$).

The inaccuracy that arises from relying on only two experimental points makes this method unsuitable for use alone. Therefore this method was normally used only for giving approximate values for T and E_m which were useful in setting up method 3.

Method 3: Application of the relationship:

$$T_o = R_o (1 + 10^{(E - E_m)/0.06})$$

(optimization of approximate values determined by method 2).


In this method several values are suggested as possibilities for the E_m of the first component. The range of values used was normally chosen by taking them around the approximate value for E_m arrived at by method 2. The value for T_1 was then recalculated from each amount of observed reduction i.e. Ro_1 , Ro_2 , Ro_3 etc. (up to a value close to the approximate T_1 value calculated by method 2) and the corresponding E values i.e. E_1 , E_2 , E_3 etc. A table was constructed showing the calculated T_1 values. Since we know that the value of T_1 is constant, the E_m value giving the least variation of T_1 must be the best approximation to the true value, and the mean value given for T_1 in this instance must also be the best approximation. If required the E_m value may be further optimized by repeating the procedure with possible values close to the first approximation.

In the present case E_m values for the calculation of T values were chosen around 50 mV (the approximate value determined by method 2) i.e. 30 mV, 40 mV, 50 mV, 60 mV and 70 mV.

Tables 3a and 3b illustrate this approach for the first 12 points on the curve of fig. 11.

The mean T value which exhibits minimum variation while satisfying all R_o values corresponds to an E_m value which is close to the mid-potential of the first component. From this it is concluded that 9.10 ± 0.10 approximates T_1 and 50 mV approximates E_{m_1} the midpoint potential of the first component. Again the figures are not far removed from the theoretical figures.

Table 3a: Resolution of Multicomponent Redox Curves

R _o	E mV	T 											
		0.50	0.60	0.80	1.20	1.60	2.20	2.90	3.70	4.60	5.50	6.30	7.10
	125	120	110	100	90	80	70	60	50	40	30	20	
30	18.90	18.90	18.90	18.70	18.30	17.70	17.10	16.30	15.40	14.50	13.50	12.60	11.90
40	13.0	13.0	13.0	13.0	12.90	12.60	12.40	12.0	11.70	11.30	10.90	10.60	10.70
50	9.0	9.10	9.10	9.10	9.10	9.10	9.10	9.10	9.10	9.20	9.20	9.30	9.30
60	6.30	6.40	6.40	6.50	6.60	6.70	6.90	7.10	7.40	7.70	8.0	8.30	8.60
70	4.40	4.50	4.50	4.70	4.90	5.10	5.40	5.80	6.20	6.70	7.20	7.70	8.10

Horizontally across the above table the mean T value is calculated at each E_m value.

Table 3b.

E _m mV	T ± S.D.	Coefficient of Variation
30	16.20 ± 2.5	15.4%
40	12.0 ± 1.0	8.3%
50	9.10 ± 0.1	1.1%
60	7.2 ± 0.8	11.1%
70	5.9 ± 1.3	22%

This method performed rather less satisfactorily under actual experimental conditions than expected from the theoretical treatment. Coefficients of variation up to 20% were observed for T values calculated for the first component. This is in marked contrast to the theoretical treatment where the coefficient of variation was only 1%. This could be due to interference from minor components and experimental scatter of points. Hence it was important that evidence for the validity of calculated parameters should always be confirmed by construction of theoretical curves which should fit the experimental curves.

The second and third methods were applied to the resolution of the first component of a two component system. The second component was found by difference from the total amount of reduction T_0 observed. In a three component system the same approach was used to resolve the first component; then after subtraction of that particular component, the procedure was repeated to resolve the second from the third component. Also in such a case two inflexion points should theoretically be seen on the curve and can be used for resolution in much the same manner as for a two components system.

v. The Method of Mixtures

The method of mixtures cannot be used for the determination of midpoint potentials of crude preparations and was hence used only for determination of the midpoint potential of purified cytochrome c_{553} .

(a) Theory: If two redox couples e.g. a cytochrome and a titrant are mixed, then at any observed potential E: (at 30°C)

$$E = E_{m_c} + \frac{0.06}{n_1} \log \frac{C^{3+}}{C^{2+}} \quad \dots\dots(1)$$

$$E = E_{m_T} + \frac{0.06}{n_2} \log \frac{T_{ox}}{T_{red}} \quad \dots\dots(2)$$

where E_{m_c} and E_{m_T} are the midpoint potentials of the cytochrome and titrant respectively. C^{3+} and C^{2+} refer to the concentrations of the oxidized and reduced species of the cytochrome; T_{ox} and T_{red} represent the concentrations of the respective species for the titrant; n_1 and n_2 represent the number of electrons transferred.

It follows that at equilibrium:

$$E_{m_c} + \frac{0.06}{n_1} \log \frac{C^{3+}}{C^{2+}} = E_{m_T} + \frac{0.06}{n_2} \log \frac{T_{ox}}{T_{red}} \dots\dots(3)$$

rearranging:

$$\log \frac{T_{ox}}{T_{red}} = \frac{n_2}{n_1} \log \frac{C^{3+}}{C^{2+}} + \frac{n_2}{0.06} (E_{m_c} - E_{m_T}).$$

This equation is of the form:

$$y = mx + c$$

Thus a plot of $\log \frac{T_{ox}}{T_{red}}$ vs $\log \frac{C^{3+}}{C^{2+}}$ yields a straight line of slope

$$\frac{n_2}{n_1} \text{ and an intercept when } \log \frac{C^{3+}}{C^{2+}} = 0 \text{ of } \frac{n_2}{0.06} (E_{m_c} - E_{m_T}).$$

Hence by carrying out the appropriate titration and plotting

$\log \frac{T_{ox}}{T_{red}}$ vs $\log \frac{C^{3+}}{C^{2+}}$, the midpoint potential for the cytochrome (E_{m_c})

can be calculated from the value of the intercept on the y axis and the midpoint potential of the titrant couple.

(b) Redox Titration

Fe^{3+} EDTA was prepared as described in section (iii) above except that Na_2 EDTA was adjusted to pH 7.0 before addition to ferric ammonium sulphate.



The cuvette contained 17 nmol cytochrome c_{553} , 0.1 mM Fe^{3+} /10 mM EDTA and 25 mM sodium phosphate buffer pH 7.0/100 mM NaCl in a final volume of 5 ml. The mixture was bubbled with argon for thirty minutes with continuous stirring before titration as well as during titration.

Titration was performed by the addition of 10 aliquots of 1 μ l each of 100 mM ferrous ammonium sulphate (100 nmol per μ l Fe^{2+}) which had been bubbled with argon for 30 minutes immediately after preparation.

The reduction of the cytochrome was followed spectroscopically by its absorption change in the region of the α -peak. The respective amounts of the reduced and oxidized species of both the cytochrome and the titrant were then calculated. The midpoint potential of the Fe^{2+}/Fe^{3+} EDTA couple at pH 7.0 is 94 mV (Kolthoff and Auerbach 1952). Since $n_2 = 1$ for Fe^{2+}/Fe^{3+} if $\log \frac{T_{ox}}{T_{red}}$ is plotted vs $\log \frac{C^{3+}}{C^{2+}}$ then

$$E_{m_c} = (\text{Intercept } y_{axis} \times 0.06) + 0.094 \quad V$$

E. Chromatographic Techniques

i. Molecular Exclusion Chromatography

All column dimensions and chromatographic conditions are given under appropriate sections. In all cases 25 mM Tris HCl pH 8.0/100 mM NaCl was used for equilibration and elution. A Mariotte flask was used as a buffer reservoir to provide a constant operating pressure.

Sepharose 4B(CL) and Sephacryl S300 were supplied preswollen by the manufacturer. Sephadex beads were allowed to swell in excess 25 mM Tris HCl pH 8.0/100 mM NaCl. The process of swelling was speeded up and air bubbles removed by heating at 75°C for 3-5 hours. The gels were then left to stand at room temperature for at least 24 hours.

Packing of all columns was performed at 4°C as described by the manufacturer (Gel filtration Theory and Practice, Pharmacia Fine Chemicals).

ii. Ion Exchange Chromatography

CM-cellulose (Whatman CM52) equilibrated with 10 mM sodium acetate buffer pH 4.5 was used for ion exchange chromatography. Chromatographic conditions and column dimensions are described in appropriate sections.

All chromatography was carried out in the cold room at 4°C.

iii. Concentration of Chromatographic Fractions on 6-Aminohexyl Sepharose

The concentration of chromatographic fractions on 6-aminohexyl sepharose is described under appropriate sections.

6-aminohexyl sepharose was prepared in the following manner:

Sepharose 4B was activated with acetonitrile (CH_3CN) under alkaline conditions. Overnight coupling to 1,6 diamino hexane (DAH) was carried out at pH 9.5. DAH was added in excess to ensure that all reactive groups on the agarose were coupled. The slurry was then washed with 0.2M acetic acid followed by 0.05M NaOH and again with 0.2M acetic acid. After thorough washing with H_2O the slurry was then equilibrated with 5 mM sodium phosphate buffer pH 7.0. All operations were carried in the fume cupboard (March et al. 1974; Shaltiel et al. 1973).

F. Amino Acid and Sequence Analysis

i. Protein Hydrolysis

Approximately 10 nmol of cytochrome were desiccated in a small pyrex test tube (7.5 cm x 1 cm ID). Water, 12M HCl and dilute thioglycollic acid were then added to give final concentrations of 6M HCl and 0.05% v/v thioglycollic acid in a volume of 300 μl . The tubes were left at -20°C for 30 minutes, then sealed under vacuum before heating in an oven for either 20 hours or 70 hours at 105°C. The contents were then dried under vacuum and redissolved in 0.2M sodium citrate pH 2.2 for application to the amino acid analyzer.

ii. Amino Acid Analysis

Amino acid analysis was carried out as described by Gardner 1981 on a Locarte floor model Mark IV amino acid analyzer with a 23 cm column of Locarte resin.

The elution programme was:

1. pH 3.25, 0.2M Na citrate, 70 minutes
2. pH 4.25, 0.2M Na citrate, 77 minutes
3. pH 9.35, 0.035 Na borate/0.12 Na₃ citrate, 100 minutes
4. regeneration with 0.2M NaOH, 20 minutes
5. re-equilibration at pH 3.25, 60 minutes.

The column temperature was initially 50° and was increased to 60° at 45 minutes. Samples were loaded in 0.2M sodium citrate pH 2.2; loading cells were filled with pH 3.25 buffer containing methanol (30% v/v). All buffers contained thiodiglycol (0.5% v/v) and Brij 35 (0.07% v/v). The ninhydrin reagent contained 2.0% (w/v) ninhydrin and 0.04% (w/v) SnCl₂ in 1:3 4 M sodium acetate (pH 5.5):methyl cellosolve.

The flow rates were 60 ml/hour for eluting buffers and 30 ml/hour for ninhydrin solution.

The analyzer was calibrated by the use of 25 nmol each of the following amino acids: Asp, Thr, Ser, Glu, Pro, Gly, Ala, CyssH, Val, Met, Ile, Leu, Tyr, Phe, His, Lys and Arg. Colour constants of these standard amino acids were calculated and from these the concentration of the amino acids of samples were obtained. Calculation of peak areas was carried out manually.

iii. N-Terminus Determination

A number of methods have been described for the determination of N-terminal residues in proteins (Allen 1981). The dansyl chloride method is the most sensitive and has been reviewed by Gray 1972.

Dansyl chloride reacts with amino groups in proteins under mildly alkaline conditions to give sulphonamide derivatives which are moderately resistant to hydrolysis in 6M HCl at 110°C, except for DNS-Pro which is 70% destroyed after overnight hydrolysis. The thiol group of cysteine, the imidazole of histidine and the hydroxyl group of tyrosine also react. However the cysteine and histidine side chain derivatives are unstable under acid hydrolytic conditions.

The principle of the method is to label the amino groups of the peptide with dansyl chloride, then to hydrolyse the peptide bonds and identify the DNS-amino acid derived from the amino terminus. The dansyl amino acids are separated by chromatography on polyamide sheets and identified by their fluorescence under U.V. light. Excess reagent is hydrolyzed to DNSOH which is also fluorescent.

(a) Dansylation of Cytochrome c₅₅₃

The dansylation of standard amino acids have been described by Gray 1972. Dansylation of cytochrome protein was carried out in the following manner:

10 nmol of cytochrome was freeze dried in a small pyrex test tube. 100 µl H₂O were added and the solution was freeze dried again. This step was repeated twice to remove traces of ammonia. 25 µl 1% w/v NaHCO₃ pH 8.5 was then added and the mixture desiccated under vacuum. 25 µl H₂O followed by 25 µl dansyl chloride (2.5 mg/ml) in acetone were then added, the tube covered with parafilm and left at room temperature overnight. The tube contents were then dried down, 100 µl 6M HCl were added, the test tube sealed and heated at 105°C for 15 hours to hydrolyse the protein. Drying in a desiccator under vacuum was then carried out and the solid material taken up in 20 µl absolute ethanol. 6 µl were taken for chromatography.

(b) Chromatography on Polyamide Sheets

Chromatography of both standard amino acids and the dansylated and hydrolyzed peptide was carried out on the two faces of a polyamide sheet (15 cm x 15 cm), essentially as described by Hartley (1970). Chromatography in the first dimension was carried out in 1.5% v/v formic acid in water. The sheet was dried, turned through 90° and chromatography in the second dimension carried out in toluene; acetic acid 9:1 v/v. The sheet was then dried, examined under U.V. and the spots identified. Rechromatography in the second dimension was carried out in the third solvent (ethylacetate:methanol:acetic acid 20:1:1 v/v) after which the plate was dried and re-examined under U.V. light.

iv. N-Terminal Sequence Determination

N-terminal sequence determination was carried out in the laboratory of Dr. R.P. Ambler of the Department of Molecular Biology, University of Edinburgh. The help and advice of Ms. M. Daniel in operating the automatic sequenator and in interpretation of chromatograms is gratefully acknowledged.

Sequencing was carried out by automated Edman degradation in a Beckman model 880B protein sequenator. The protein was subjected to 12 cycles of reaction with phenylisothiocyanate essentially as described by Niall 1973. 5% phenylisothiocyanate in heptane was used for coupling, followed by dimethyl benzylamine pH 9.4 in normal propanol:H₂O (12:40:48 v/v) as coupling buffer. Heptafluorobutyric acid was used for cleavage, benzene for washing and butyl chloride for extraction.

The amino acid derivatives were converted to the phenyl thiohydantoin derivatives by heating in the presence of 1M HCl for 10 minutes at 80°C. The fractions were then extracted in ethylacetate,

the extraction procedure being repeated twice. The ethyl acetate was then blown off to dryness under nitrogen. The residual material was dissolved in a minimum volume of ethyl acetate and applied to a fluorescent silica gel plate (20 cm x 20 cm) together with standard amino acid derivatives.

Butyl acetate:formamide:propionic acid:H₂O (60:8:2:1 v/v) were shaken together and the top layer was taken for the chromatography. After chromatography the plates were examined under U.V. light. Fluorescent spots were marked with a pencil. The plate was then dipped in 2% ninhydrin in acetone, dried at room temperature and heated at 105° for 2-3 minutes. The spots were identified by their mobilities and colour.

G. Polyacrylamide Gel Electrophoresis

i. Materials

- a) 30% w/v acrylamide/bisacrylamide: 29.1 g acrylamide and 0.9 g bisacrylamide in 100 ml aqueous solution. The solution was immediately filtered through Whatman No. 1 filter paper and stored at 4°C in dark bottle.
- b) TEMED:N,N,N',N' tetramethylene diamine.
- c) 10% w/v ammonium persulphate freshly prepared.
- d) 1M sodium acetate buffer pH 4.5.
- e) 0.2M sodium phosphate buffer pH 7.0 0.2% w/v in SDS (sodium dodecylsulphate).

ii. Preparation of Gels

10% cylindrical gels (8 x 0.6 cm) were made by polymerization of a solution containing 10%, w/v acrylamide/bisacrylamide, 0.5% v/v TEMED, and 0.1% w/v ammonium persulphate. The solution contained 0.1% w/v SDS and 0.1M sodium phosphate buffer pH 7.0 in the case of SDS gels and 0.1M sodium acetate pH 4.5 in the case of native gels.

iii. Protein Denaturation

For SDS gel electrophoresis proteins were denatured by heating in a water bath at 75°C for 15 minutes in the presence of 1% w/v SDS, 5% glycerol and 0.01M sodium phosphate buffer pH 7.0. Mercaptoethanol was omitted as a disulphide reductant since it bleaches cytochromes and prevents their detection by subsequent scanning of the gels at 410 nm.

iv. Electrophoretic Conditions

SDS gels were prerun at 3 mA/gel for one hour. SDS gels were run at 8 mA/gel for 6 hours. Native gels were run at 5 mA/gel for 4 hours.

v. Gel Staining

0.2% w/v Coomassie Brilliant Blue R in methanol:H₂O:acetic acid: 5:5:1 v/v was used as a protein stain. Methanol:H₂O:acetic acid 2.5:6.8:0.7 v/v was used for destaining.

vi. Scanning of Gels

Unstained gels were scanned for 410 nm absorbing material. Gels stained for protein were scanned at 560 nm. Scanning was carried out in a Unicam-Gilford gel scanner.

CHAPTER III

REDOX POTENTIOMETRIC TITRATIONS

A. Introduction

Redox titrations were performed on the crude fractions of the cell extract of Campylobacter sputorum subspecies mucosalis with the following objectives in mind:

- a) to determine the number and midpoint potentials of the C-type cytochromes in the crude cell extract;
- b) to determine the distribution of these cytochromes in the high speed (100,000 g) supernatant and sedimentable material;
- c) to establish ultimately that isolated and purified (or partially purified) cytochromes correspond in midpoint potentials to those identified by redox potentiometry of the crude preparations.

i. General

Since redox proteins react poorly with a metal electrode, one or more of the mediators shown in table 2 were added to the reaction mixture at a final concentration of 10 μM in the cuvette, except for Fe^{3+} EDTA which was used at a concentration corresponding to 20 μM Fe^{3+} / 400 μM EDTA. Such mediators mediate between the protein and the electrode, but would also "buffer" the titration. Inspection of the Nernst equation shows that it is similar to the Henderson-Hasselbalch equation and hence large changes in the reduced (or oxidized state) in the region of the midpoint potential of any one particular redox couple would lead to relatively small changes in the observed potential i.e. there is resistance to change as one proceeds from one extremity of titration towards the midpoint potential of that redox couple, and by analogy to pH titrations one can speak of "redox buffering".

ii. Reasons for the procedure finally adopted during redox titrations

The chosen concentrations of mediators was a compromise between achieving good redox buffering and stability of the potentials, and the undesirable necessity of adding larger and larger amounts of titrants to get past the midpoint potential of any one specific mediator. The mediators are also chromophores and the spectroscopic changes accompanying reduction were also a determining factor in the choice of final mediator concentration used. It is desirable to "buffer" any reaction mixture throughout the whole range of titration by the use of mediators with midpoint potentials covering the range of titration at approximately 50 mV intervals. In practice this was governed by the commercial availability of such mediators, and any undesirable effect, such as base line shift, they may have on the titration.

In initial experiments, in order to achieve anaerobic conditions inside the cuvette, argon was bubbled into the solution 15 minutes before titration. This procedure however led to frothing, progressive denaturation and precipitation, and consequent loss of protein. It was hence abandoned in favour of bubbling the cuvette contents without the cytochrome for 15 minutes, followed by quick addition of the cytochrome and blowing of argon over the surface of the solution for one hour before titration was performed. The reaction mixture was adequately stirred throughout the equilibration with argon and the titration. All titrants were also continuously equilibrated with argon.

Two main difficulties were encountered during titrations. The base line of the titration would continuously shift throughout the titration, due to the reduction of the mediators as well as other physiological species such as quinones and iron sulphur proteins in

the crude preparations. Protein precipitation during titration would also lead to the same effect. This difficulty was circumvented by determining an isosbestic point of the crude preparations (544 nm), setting the spectrophotometer to read at a certain arbitrarily chosen value at that particular wavelength, and adjusting the reading to the same value each time the spectrum of the reduced or partially reduced cytochrome was recorded. Such a procedure is in effect equivalent to the use of a dual wavelength spectrophotometer, which subtracts the absorbance at a certain predetermined wavelength from that at another reference wavelength.

The second difficulty encountered was a continuous drift of the potential to more positive values in titrations in which sodium dithionite was used as a titrant. It was suspected that an undefined sodium dithionite breakdown product could receive electrons from the electron transport system and consequently the cytochromes would reoxidize and the potential would drift to more positive values. Such breakdown products were reported by Mayhew 1978. In the case of Campylobacter sputorum subspecies mucosalis this phenomenon suggests the presence of an oxidase utilizing a sulphur compound and may be of physiological importance.

Sodium dithionite was hence replaced as a titrant with either NADH or sodium formate. In the latter case it was essential that any traces of oxygen were removed from the system at the initial stages of the titration. Niekus et al. 1980a & b reported formate dehydrogenase to be inactivated under aerobic conditions at room temperature, particularly in the presence of its substrate, and that formate oxidation by oxygen leads to the formation of H_2O_2 . Since the redox couple $\text{H}_2\text{O}_2/\text{O}_2$ has a high midpoint potential (+300 mV), its presence in the redox system may lead to reoxidation of the

cytochromes and hence drift in potential. Therefore titrations were initiated with NADH or ascorbate as titrants down to a potential of approximately 100 mV, after which sodium formate was used.

iii. Calibration of the Redox Electrode

While Dutton 1978 gives a standard potential of 220 mV at 25°C for the Ag/AgCl electrode, Bates 1954 (referred to in Wood 1980) gives a value of 198 mV. In view of this discrepancy it was important that determination of the reference electrode potential be carried out.

The potential of the reference electrode was initially measured by full titration against the Fe-EDTA couple and the value obtained was checked by a two point titration before each redox titration of cytochromes was performed.

Calibration of the Ag/AgCl electrode was performed as described under materials and methods. A result for such a titration is shown in Fig. 12. The mean and standard deviation of 16 such determinations over a period of six months was 198.7 ± 5.4 mV in good agreement with the value obtained by Bates 1954.

B. Redox Potentiometric Titrations

i. Redox Potentiometry of the Cell Extract

Redox potentiometry of the cell extract was carried out as described in the legend to fig. 13 - the spectra of the titration.

A non-linear and complex curve was obtained when the observed potentials were plotted vs $\log \frac{(\text{ox})}{(\text{red})}$ (fig. 14). By the methods outlined in chapter II the complex curve could be resolved into 3 components of mid potentials + 90 mV, - 125 mV and -305 mV (fig. 15). Each component contributed one third of the total reducible material of 108 nmol extracted per g cells.

The theoretical curve constructed from the resolved components (fig. 14) fits the experimental results rather well.

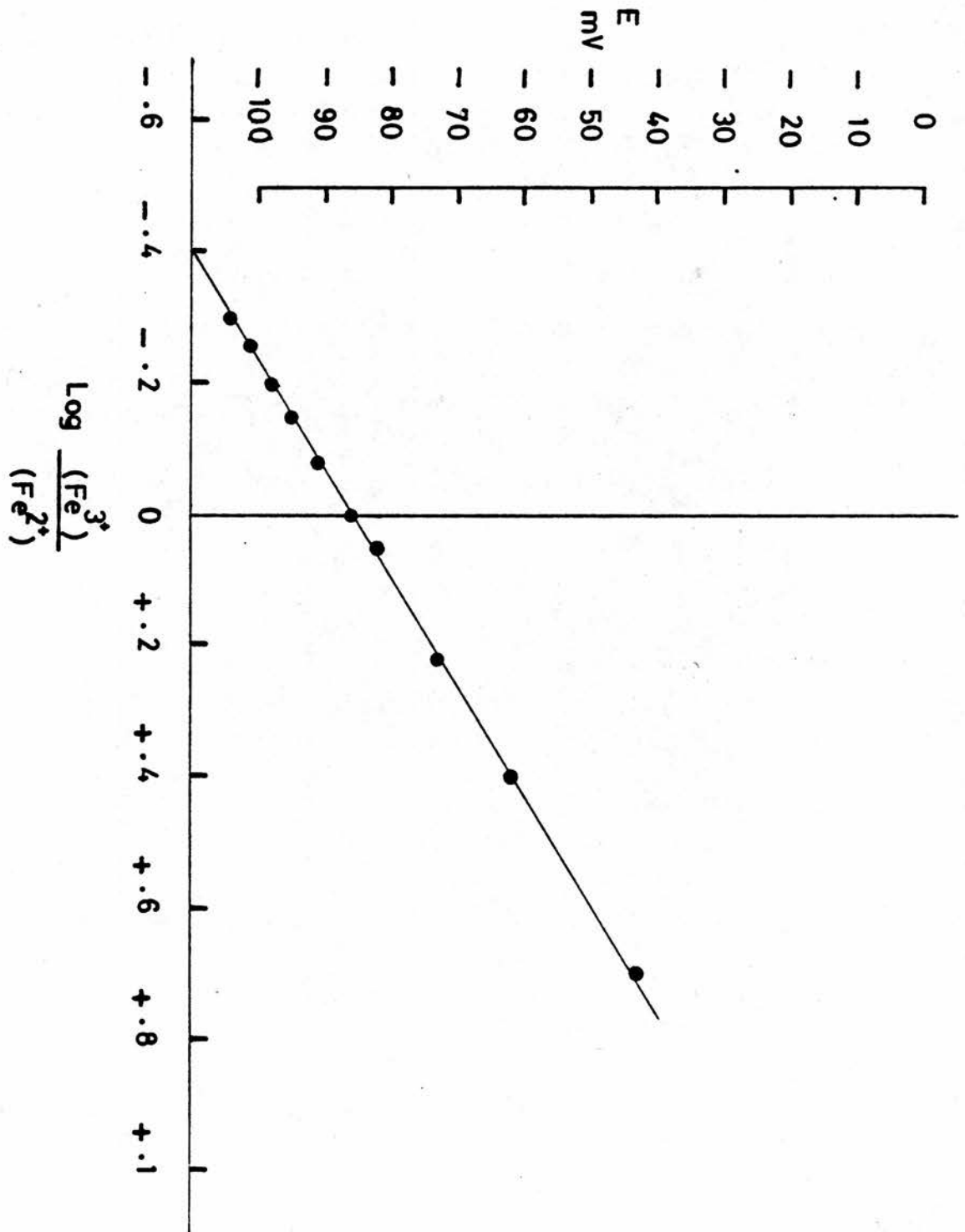
Fig. 12: Calibration of Ag/AgCl Electrode

Fig. 12: Calibration of Ag/AgCl Electrode

Electrode calibration was carried out against the $\text{Fe}^{2+}/\text{Fe}^{3+}$ EDTA couple as described under methods. The line obtained is a best fit and gives a 60 mV slope. The electrode potential is obtained from:

$$-87 = 112 - \text{SEP Ag/AgCl}$$

$$\therefore \text{SEP Ag/AgCl electrode} = 199 \text{ mV}$$

SEP = standard electrode potential.

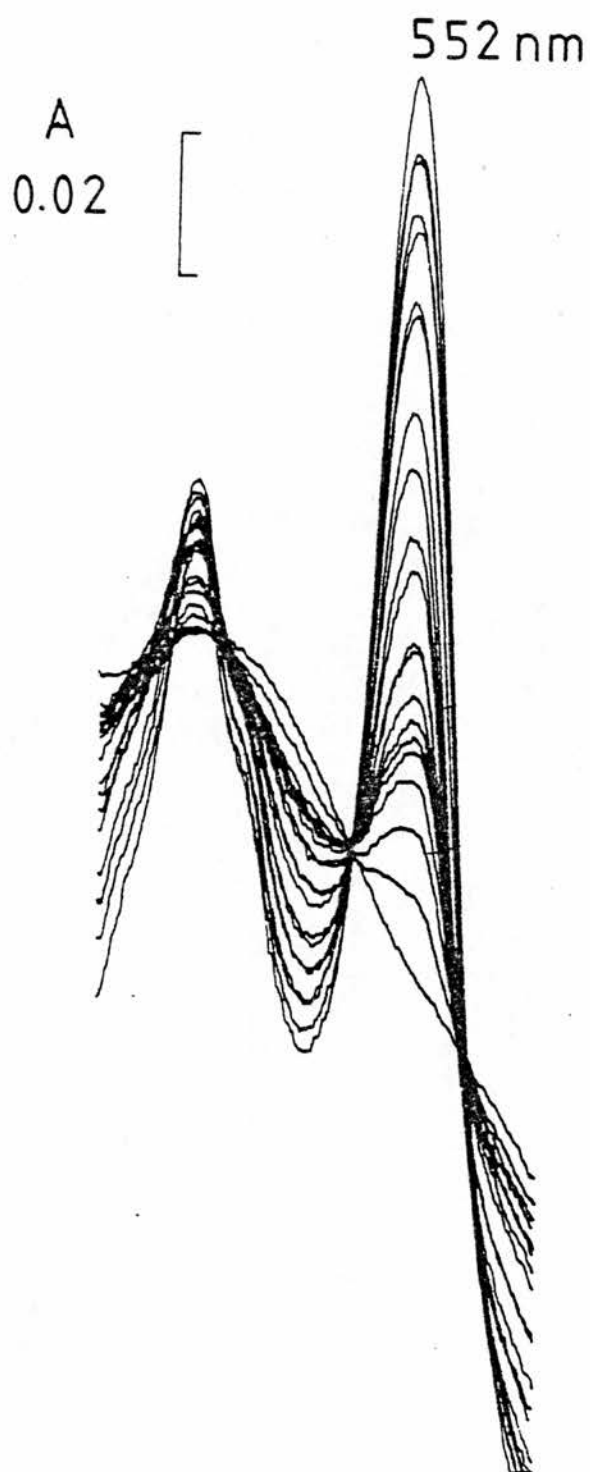
Fig. 13: Redox Titration Cell Extract

Fig. 13: Redox Titration Cell Extract

1 gram cells was extracted in 3 ml 25 mM sodium phosphate buffer pH 7.0/100 mM NaCl as described under extraction procedure. 36 nmol of cytochrome were titrated in presence of 10 μM each of PES, PMS, HNQ, BV, DQ, DAD and FMN. 20 μM Fe^{3+} as Fe^{3+} EDTA (Fe^{3+} : EDTA = 1:20 mol/mol) was also included as a redox mediator. Final volume was 4 ml. Titration was initiated with 100 mM ascorbate down to a potential of 50 mV, then completed with 50 mM Na formate.

Spectra were recorded as outlined in text.

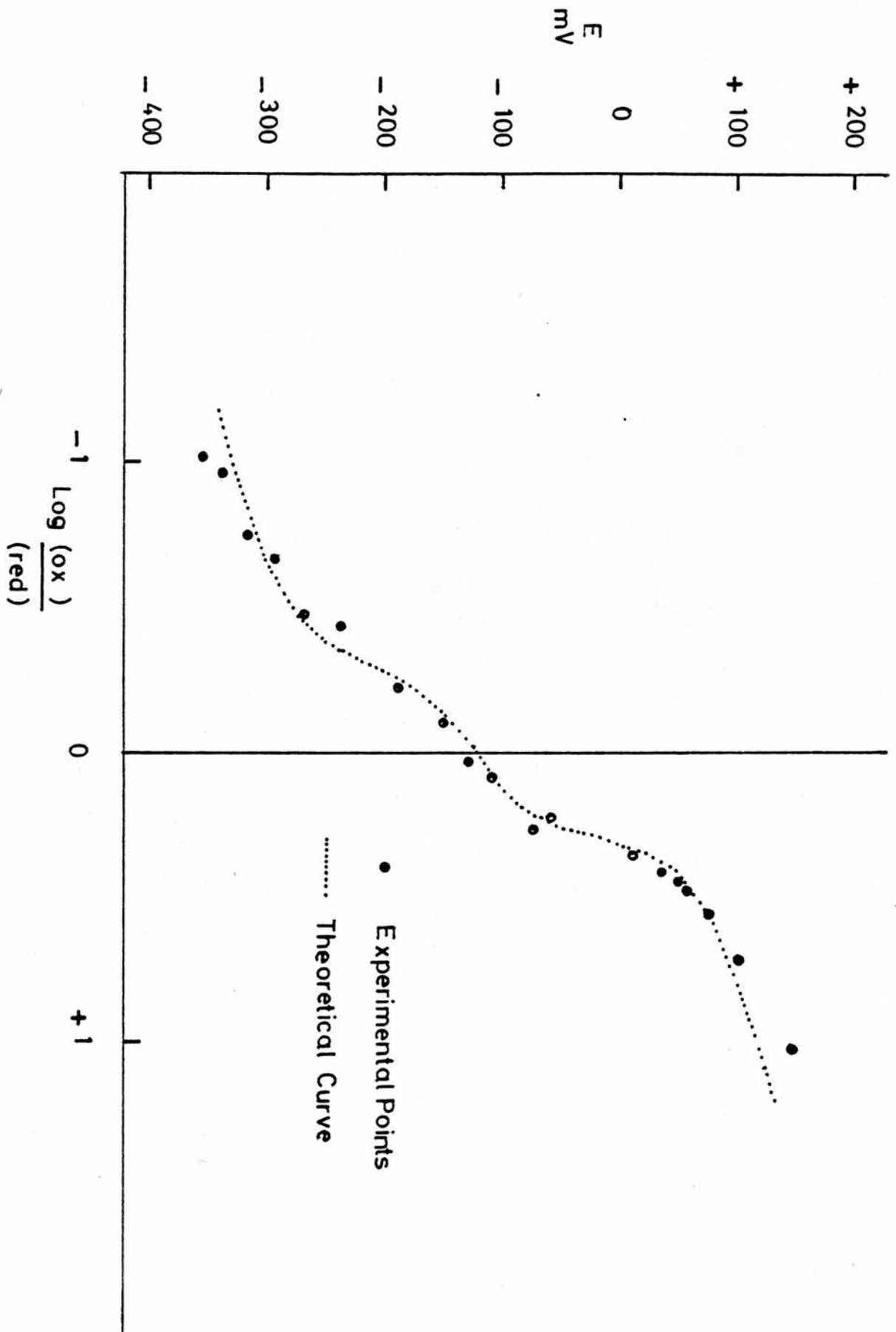
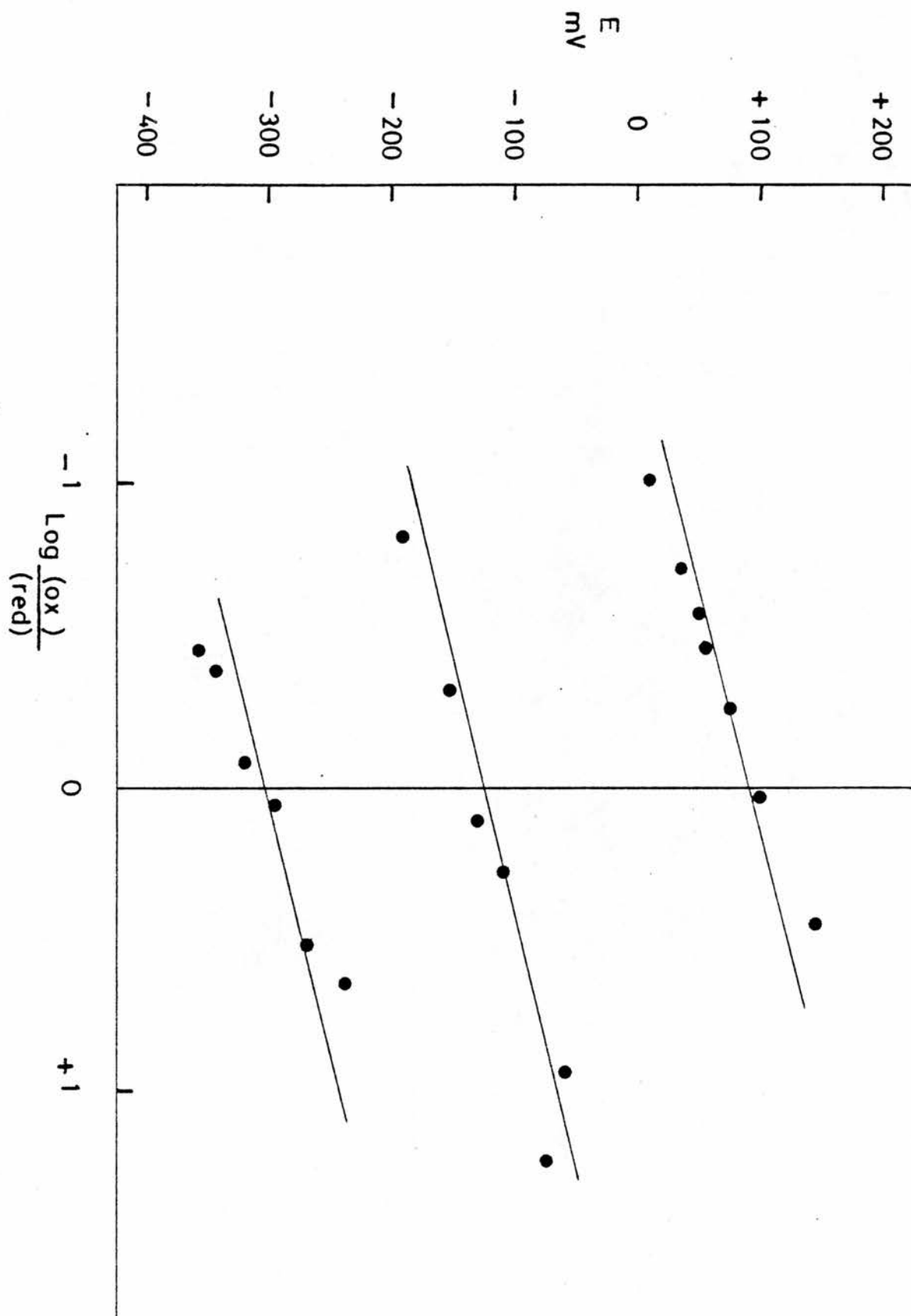
Fig. 14: Redox Titration of Cell Extract

Fig. 14: Redox Titration of Cell Extract

Experimental points correspond to those obtained from the spectra in fig. 13 and the observed redox potentials. The theoretical curve was constructed from parameters of the resolved components (fig. 15).

Fig. 15: Resolved Components - Redox Titration Cell Extract

Resolved components were separated by means of mathematical approaches outlined in methods. Lines are of 60 mV slope.

ii. Redox Potentiometry of the High Speed (100,000 g) Supernatant

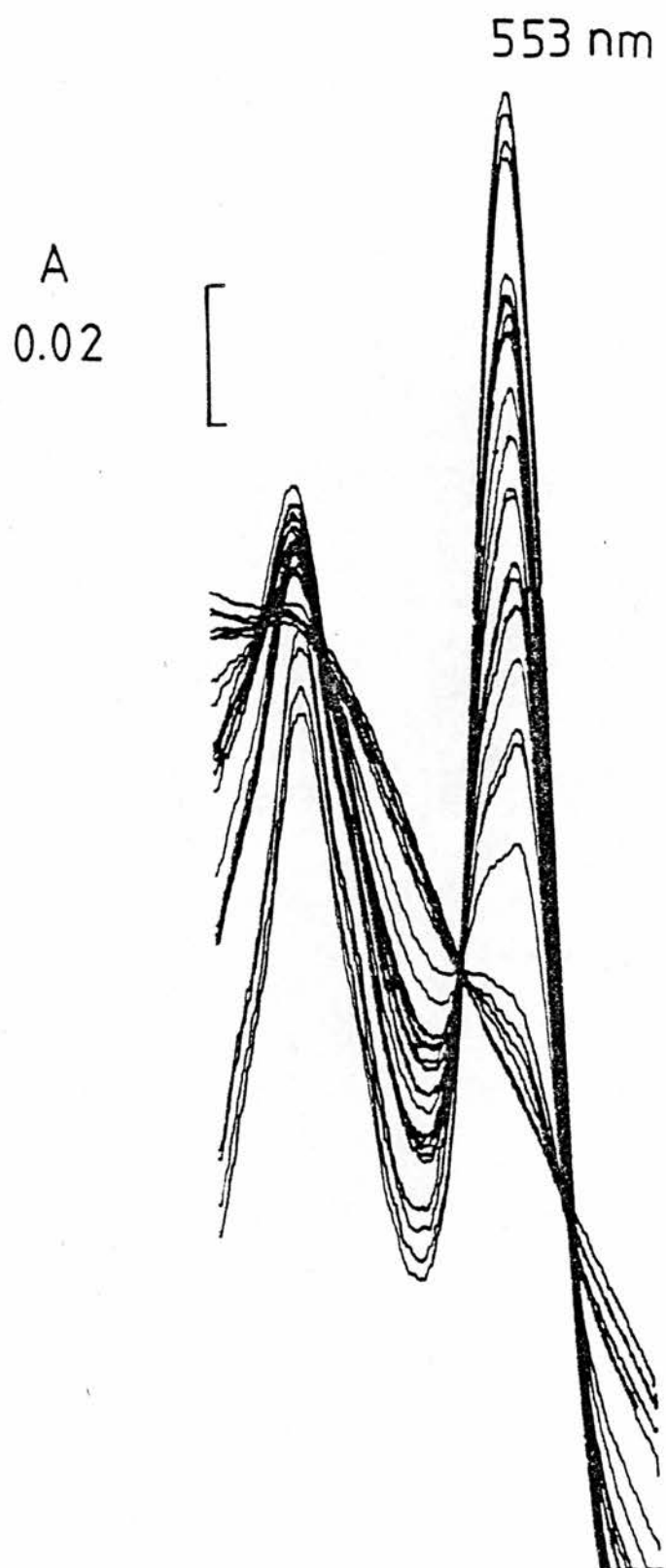
Redox potentiometry of the high speed supernatant was carried out as described in the legend of fig. 16. A non-linear complex curve was again obtained when the data were plotted (fig. 17). The curve could be resolved into 3 components of midpoint potentials of +95 mV, -137 mV and -310 mV (fig. 18), with a contribution of 19, 10, and 5 nmol reducible material per g cells respectively. The theoretical curve constructed from the resolved components shows a good fit to the experimental results (fig. 17).

iii. Redox Potentiometry of the High Speed (100,000 g) Sedimentable Material

Redox potentiometry of the high speed (100,000 g) sedimentable material was carried out as described in the legend of fig. 19. The complex experimental curve (fig. 20) could be resolved into 3 components of midpoint potentials of +80 mV, -125 mV and -320 mV (fig. 21). The contribution of these to total reducible material was 15 nmol, 27 nmol and 30 nmol per g cells. A good fit was obtained between the experimental results and the theoretical curve constructed from the resolved components (fig. 20).

iv. Conclusions and Discussion

The spectra for the titration of the high speed supernatant (fig. 16) clearly show a prominent asymmetric α -peak with an absorption maximum at 553 nm. The asymmetry is not apparent at the initial points of titration, becomes striking within the first third of the titration and then appears to be progressively lost merging into a symmetric peak. This suggests the presence of a positive midpoint potential cytochrome c with an asymmetric α -peak in the 100,000 g supernatant. This cytochrome will hence forth be referred to as cytochrome c_{553} .

Fig. 16: Redox Titration of the High Speed Supernatant

Figs. 16 & 19: Redox Titration of High Speed (100,000 g)

Supernatant and Sedimentable Material

2 gram cells were extracted in 6 ml 25 mM sodium phosphate buffer pH 7.0/100 mM NaCl as described under extraction procedure. The cell extract was then centrifuged at 30,000 rpm (100,000 g) for 15 hours at 4°C. The sedimented material was resuspended in the same buffer and centrifuged at 10,000 rpm (7,000 g) for 5 minutes to remove turbidity. NADH (150 mM) was used for titration of the supernatant throughout and for initial titration of the 100,000 g sedimentable material down to 100 mV potential after which 50 mM sodium formate was used. PES, PMS, HNQ, BV, DQ, DAD, and FMN were used as redox mediators, all at final concentrations of 10 μ M. Fe^{3+} EDTA (Fe^{3+} :EDTA: 1:20 mol/mol) was included as a redox mediator at a concentration corresponding to 20 μ M Fe^{3+} .

Spectra were recorded as outlined in text.

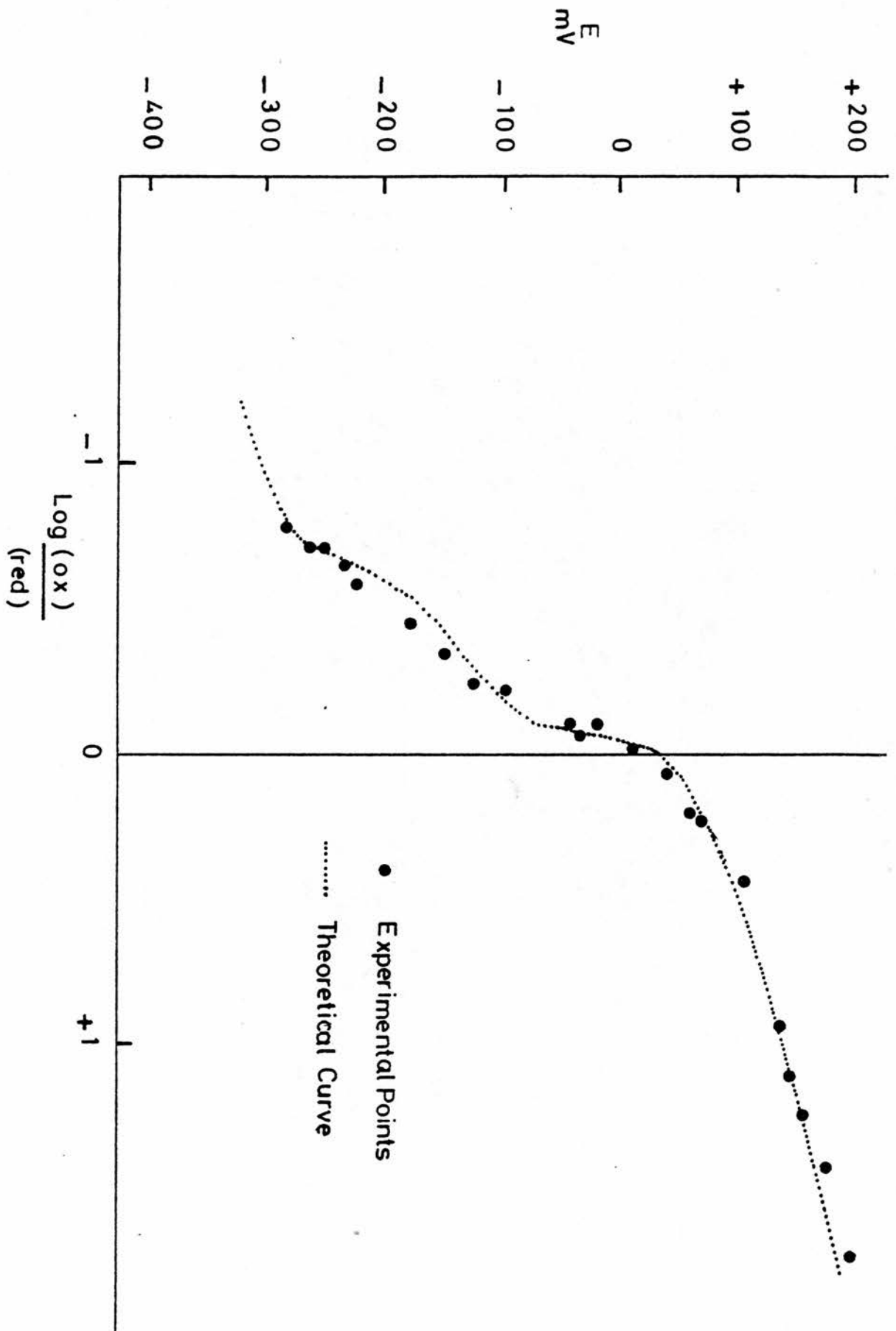
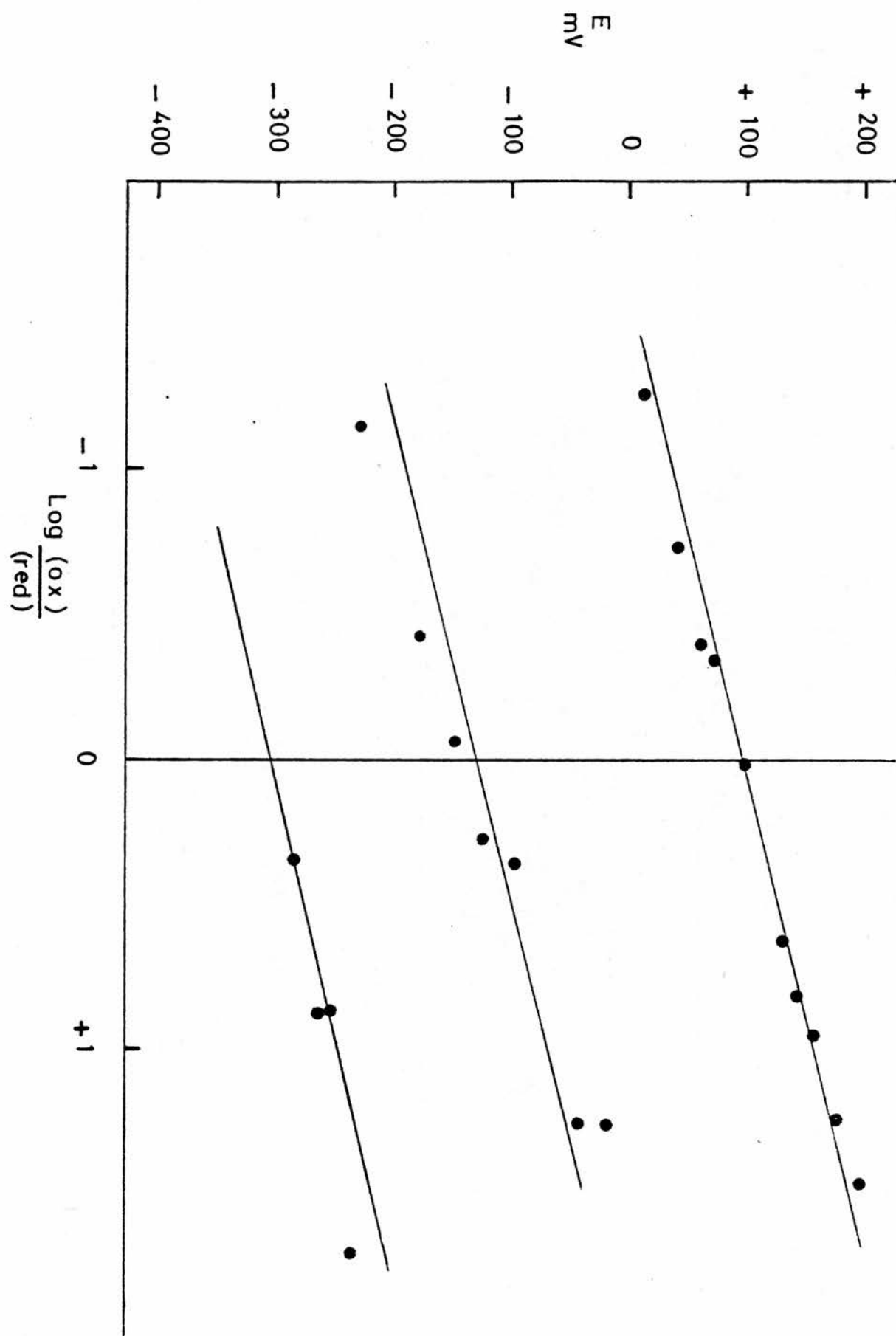
Fig. 17: Redox Titration - High Speed Supernatant

Fig. 17: Redox Titration - High Speed Supernatant

Experimental points were obtained from the spectra of fig. 16 and the observed redox potentials. The theoretical curve was constructed from parameters of the resolved components (fig. 18).

Fig. 18: Resolved Components: Redox Titration High Speed Supernatant

Resolved components were separated by approaches outlined in methods. The lines are of 60 mV slope.

Fig. 19: Redox Titration - 100,000 g Sedimentable Material

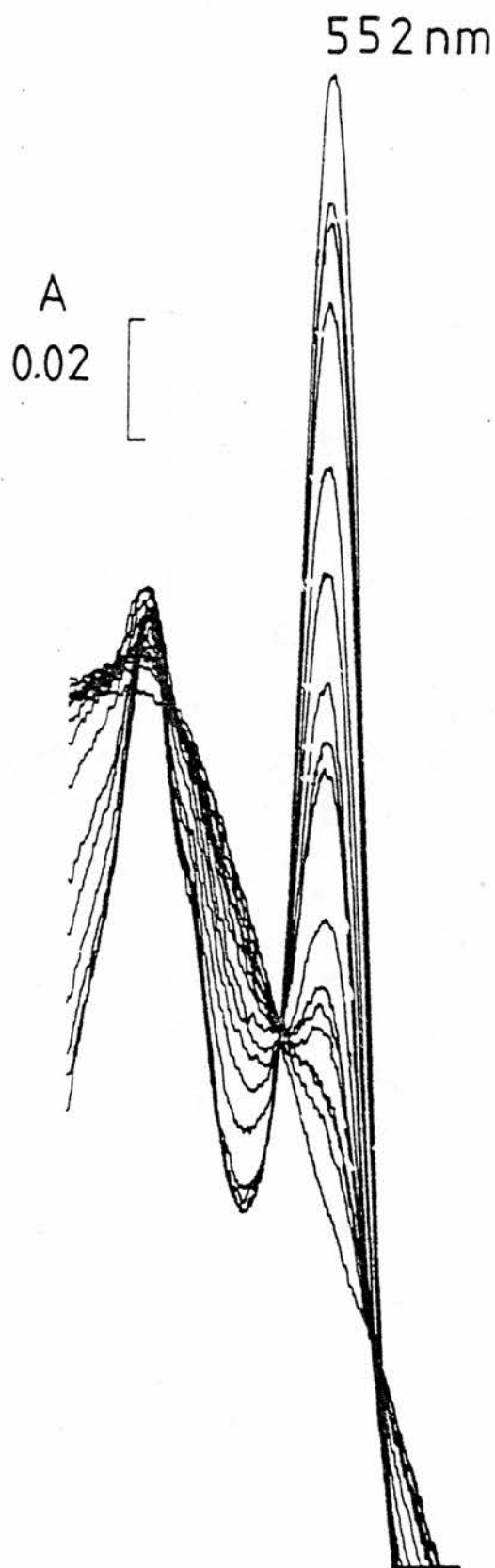


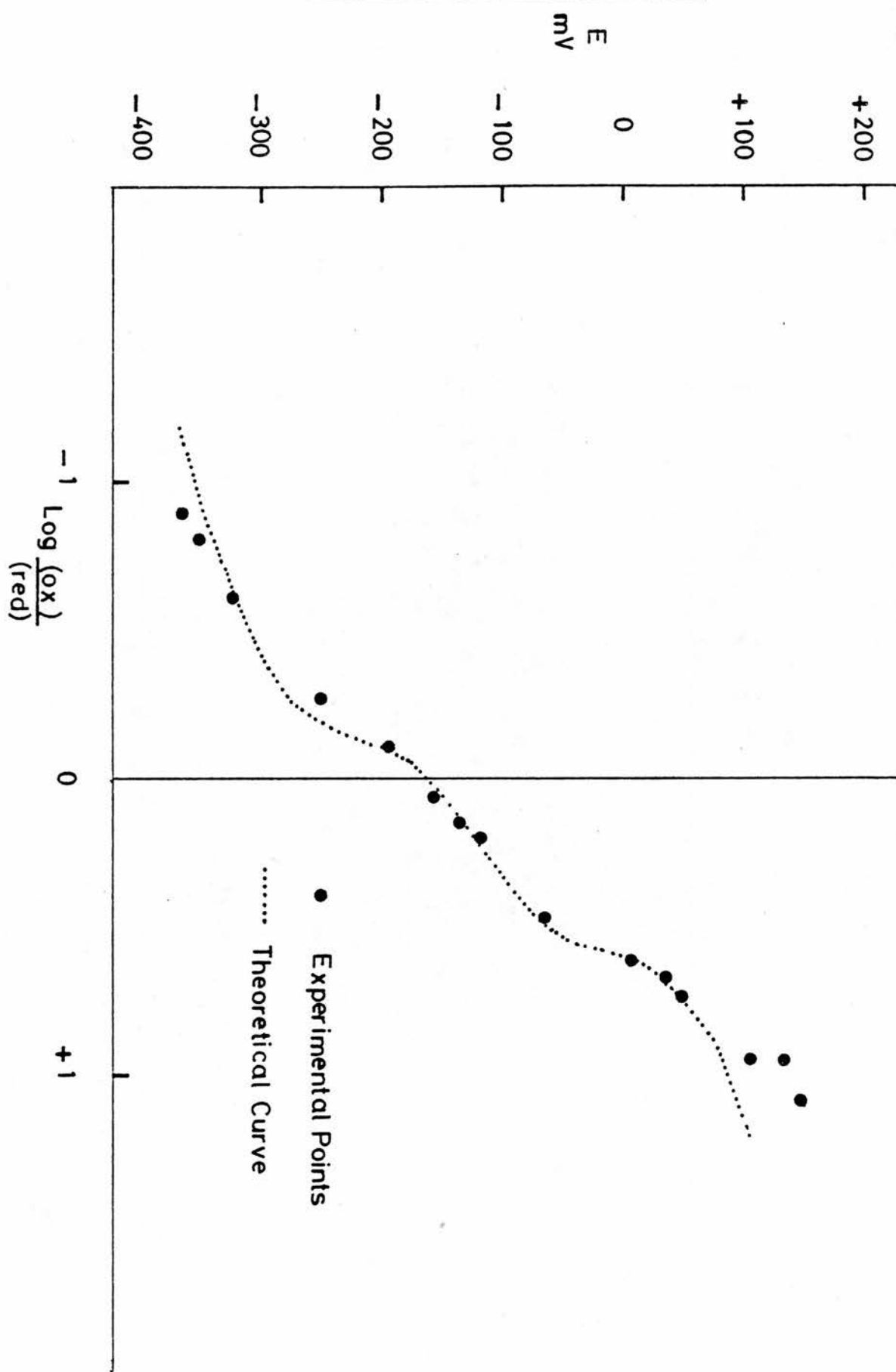
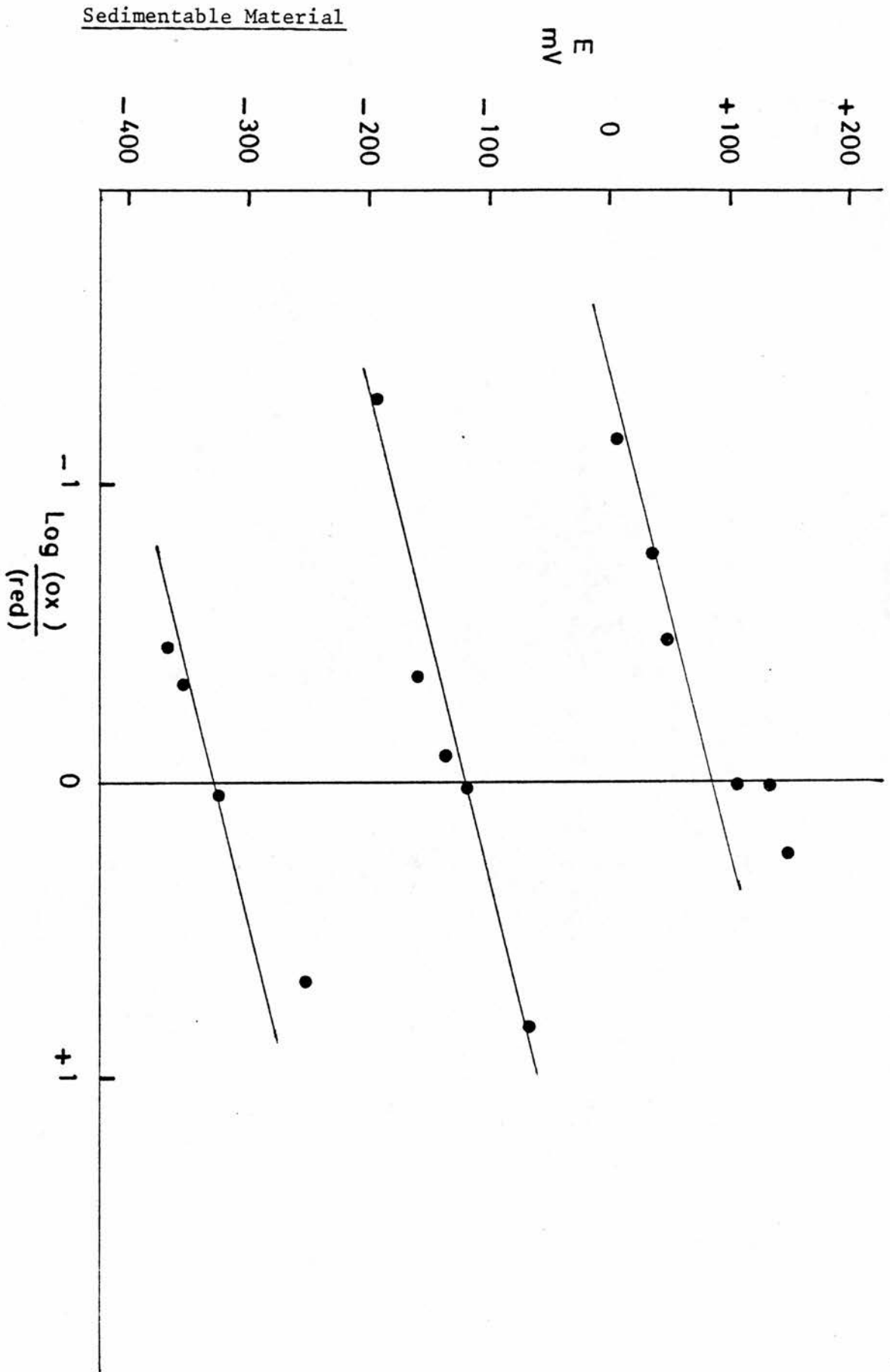
Fig. 20: Redox Titration - 100,000 g Sedimentable Material

Fig. 20: Redox Titration - 100,000 g Sedimentable Material

Experimental points were obtained from the spectra (fig. 19) and the corresponding observed redox potentials. The theoretical curve was constructed from the resolved components (fig. 21).

Fig. 21: Resolved Components - Redox Titration 100,000 g

The resolved components were separated mathematically as outlined in methods. The lines are of 60 mV slope.

Asymmetry of the α -peak is difficult to identify in the titrations of the cell extract (fig. 13) and is not apparent in the high speed sedimentable material (fig. 19), in spite of the presence of a component of positive midpoint potential. It is therefore proposed that the presence of a cytochrome c with a positive midpoint potential and a symmetric α -peak in the 100,000 g sedimentable material masks the asymmetric α -peak of cytochrome c_{553} in the crude cell extract.

Taken together the spectral observations and the results of redox potentiometry (table 4) suggest the presence of at least four cytochromes c in the crude cell extract of Campylobacter sputorum subspecies mucosalis. The presence of cytochrome c_{553} as a soluble and dominant component in the 100,000 g supernatant and the distinctiveness of its asymmetric α -peak make it a good candidate for purification and characterization.

Two cytochromes c with negative midpoint potentials ($E_m - 125$ mV and $E_m - 320$ mV) are predominant in the 100,000 g sedimentable fraction (table 4). Cytochromes c with such low midpoint potentials have seldom been described in the literature. The occurrence and properties of such cytochromes and the involvement of both positive and negative midpoint potential cytochromes c in bacterial electron transport chains will be treated in greater detail in the main discussion of work presented in this thesis (chapter VI).

Table 4: Summary Redox Potentiometric Data

(i) Midpoint potentials of components

Fraction	Midpoint Potentials mV		
	Components		
	I	II	III
A. Cell extract	+90	-125	-305
B. 100,000 g supernatant	+95	-137	-310
C. 100,000 g sedimentable fraction	+80	-125	-320

(ii) Relative contribution of components

Fraction	nmol/g Cells		
	Components		
	I	II	III
A. Cell extract	36	36	36
B. 100,000 g supernatant	19	10	5
C. 100,000 g sedimentable fraction	15	27	30
Sum B + C	34	37	35

CHAPTER IV

PURIFICATION AND PROPERTIES OF CYTOCHROME C c₅₅₃

A logical approach to the confirmation of the validity of the proposals put forward as a result of redox potentiometric data in chapter III is the isolation, purification and characterization of the cytochromes predicted to be present in the cell extract of Campylobacter sputorum subspecies mucosalis.

This chapter deals with the purification and properties of cytochrome c_{553} . This cytochrome, with a positive midpoint potential, was identified potentiometrically in the 100,000 g supernatant as the dominant cytochrome c in this fraction. Furthermore it was proposed that this cytochrome has an asymmetric α -peak with a maximum at 553 nm; hence the designation.

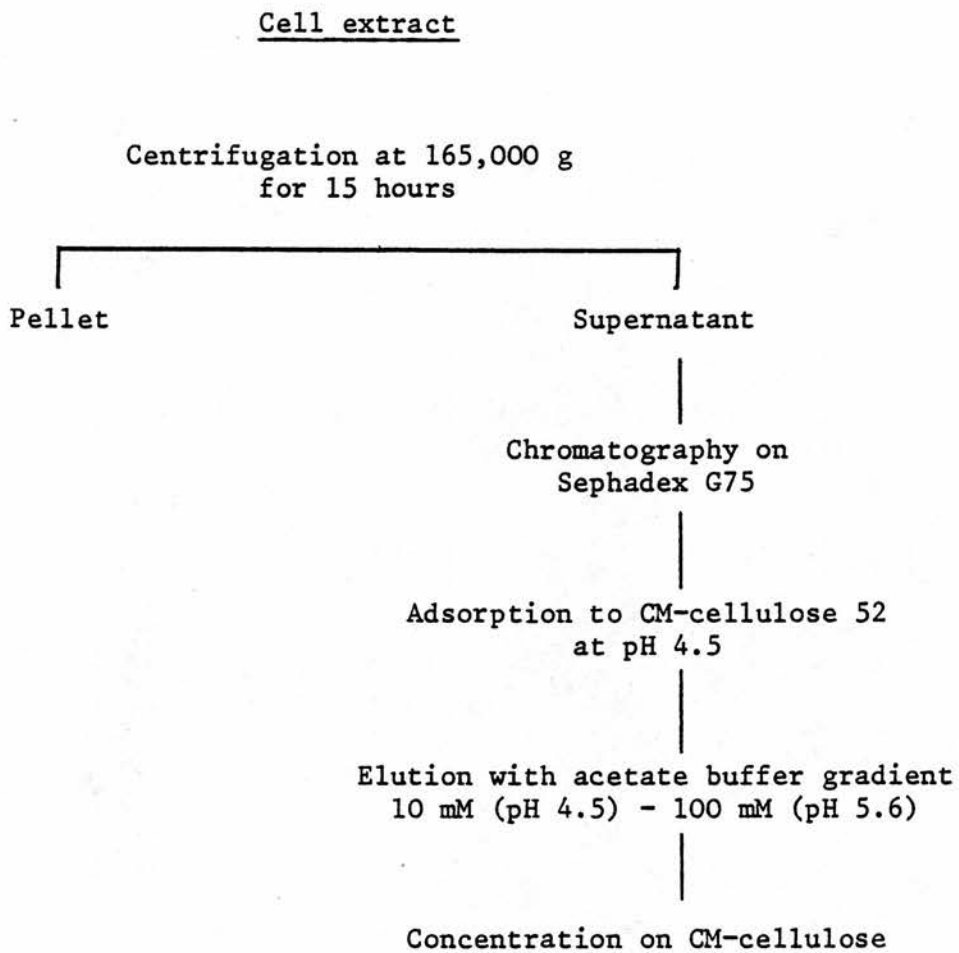
The object of work described in this chapter is two fold:

- (i) to obtain the cytochrome in a potentiometrically homogeneous state and confirm that the midpoint potential proposed for it from redox potentiometric titrations of the crude preparations agrees with that obtained for the separated preparation.
 - (ii) to obtain the protein in a homogeneous state so that characterization of the cytochrome with respect to spectra, amino acid composition, molecular weight and other physicochemical properties is possible.
- The purification procedure of cytochrome c_{553} is summarized in fig. 22.

A. Extraction and Purification of Cytochrome c_{553}

i. Extraction

3-4 g cells were extracted in a volume not exceeding 15 ml of 25 mM sodium phosphate buffer pH 7.0/100 mM NaCl as described under the extraction procedure outlined in chapter II. The crude extract so obtained was then centrifuged at 165,000 g (50,000 rpm; MSE "Superspeed 65" centrifuge) for 15 hours at 4°C. Subsequent steps were carried on the 165,000 g supernatant.

Fig. 22. Purification Scheme-Cytochrome c₅₅₃

ii. Molecular Exclusion Chromatography

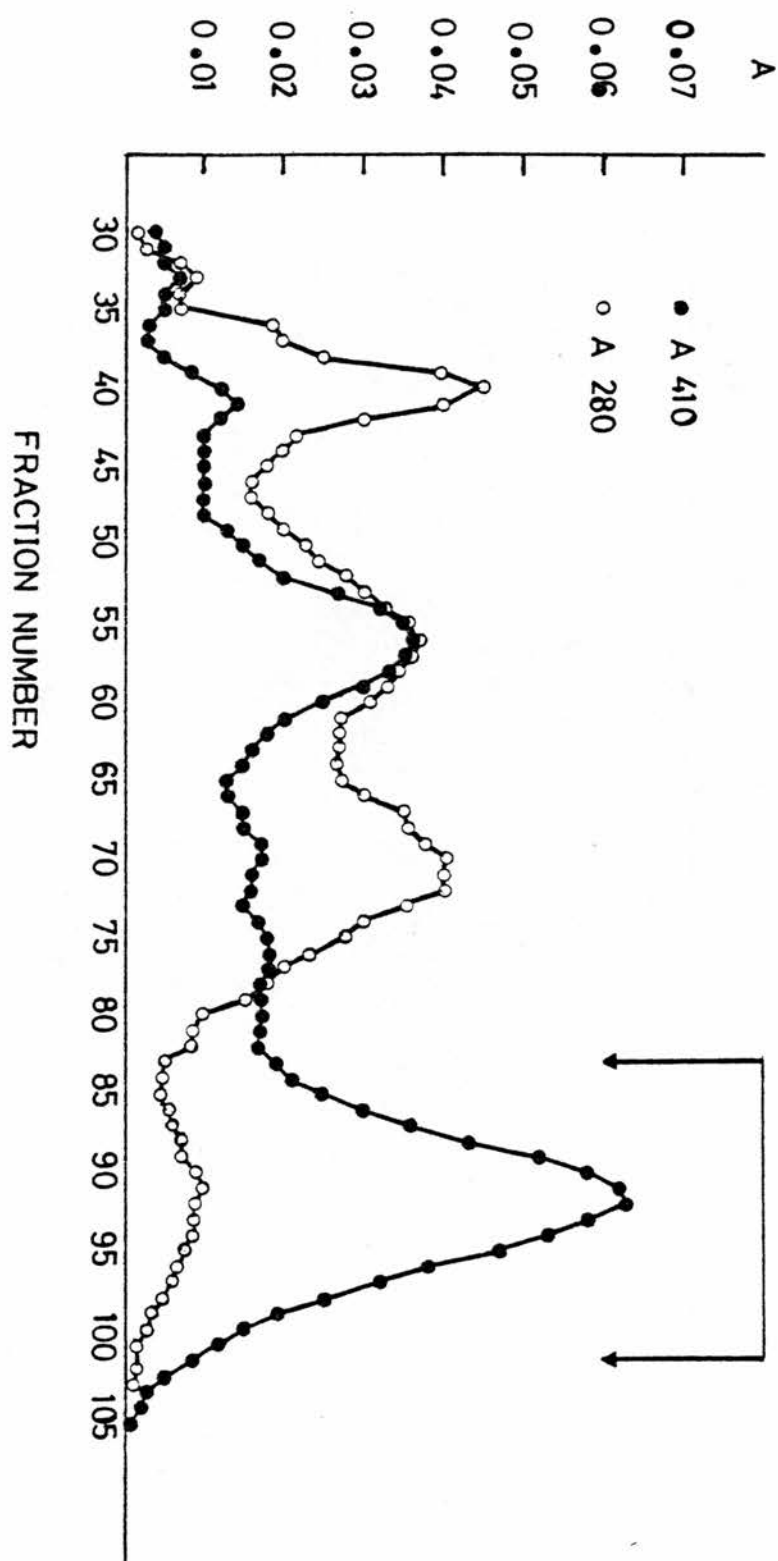
The supernatant from the 165,000 g centrifugation of the crude cell extract was applied to a sephadex G75 column of 90 x 3.5 cm dimensions. 25 mM Tris HCl pH 8.0/100 mM NaCl was used for equilibration and elution at 4°C. The void volume of the column was determined using blue dextran (MW 2×10^6), while calibration of the column was carried out with horse heart cytochrome c, ovalbumin and bovine serum albumin as molecular weight markers. The flow rate was 0.40 ml per minute.

The elution pattern was monitored at 410 nm and 280 nm (fig. 23). A major chromatographic peak absorbing at 410 nm dominates the profile and corresponds to a molecular weight of approximately 11,000 (fig. 35). Fractions corresponding to this peak were pooled and frozen at -40°C. Three such column preparations were usually carried out and combined before the next stage of purification.

iii. Carboxymethyl Cellulose Ion Exchange Chromatography

Ion exchange chromatography was carried out in a column of 18 x 2.5 cm dimensions of carboxymethyl cellulose (Whatman CM 52). The column was equilibrated with 500 ml of 10 mM sodium acetate pH 4.5 at 4°C.

The pooled sephadex G75 fractions were adjusted to pH 4.5 by titration with 1M acetic acid, dialyzed against 3 changes of 10 volumes of 10 mM sodium acetate buffer pH 4.5 over a period of 24 hours, and then applied to the CM-cellulose column. Elution of the cytochrome was performed by the use of a pH (4.5-5.6) and ionic strength (10 mM-100mM) gradient of 2 x 500 ml volume. The elution profile is shown in fig. 24 and shows a peak of protein (fractions 41-44); a trace amount of 410 nm absorbing material (fractions 40-51) is also observed. Such minor chromatographic peaks are frequently observed

Fig. 23: Molecular Exclusion Chromatography of 165,000 gSupernatant

Molecular exclusion chromatography of the 165,000 g supernatant was carried out as described in text. The arrows indicate fractions each of 5.6 ml pooled for further treatment.

Fig. 24: CM-Cellulose Chromatography of Cytochrome c₅₅₃

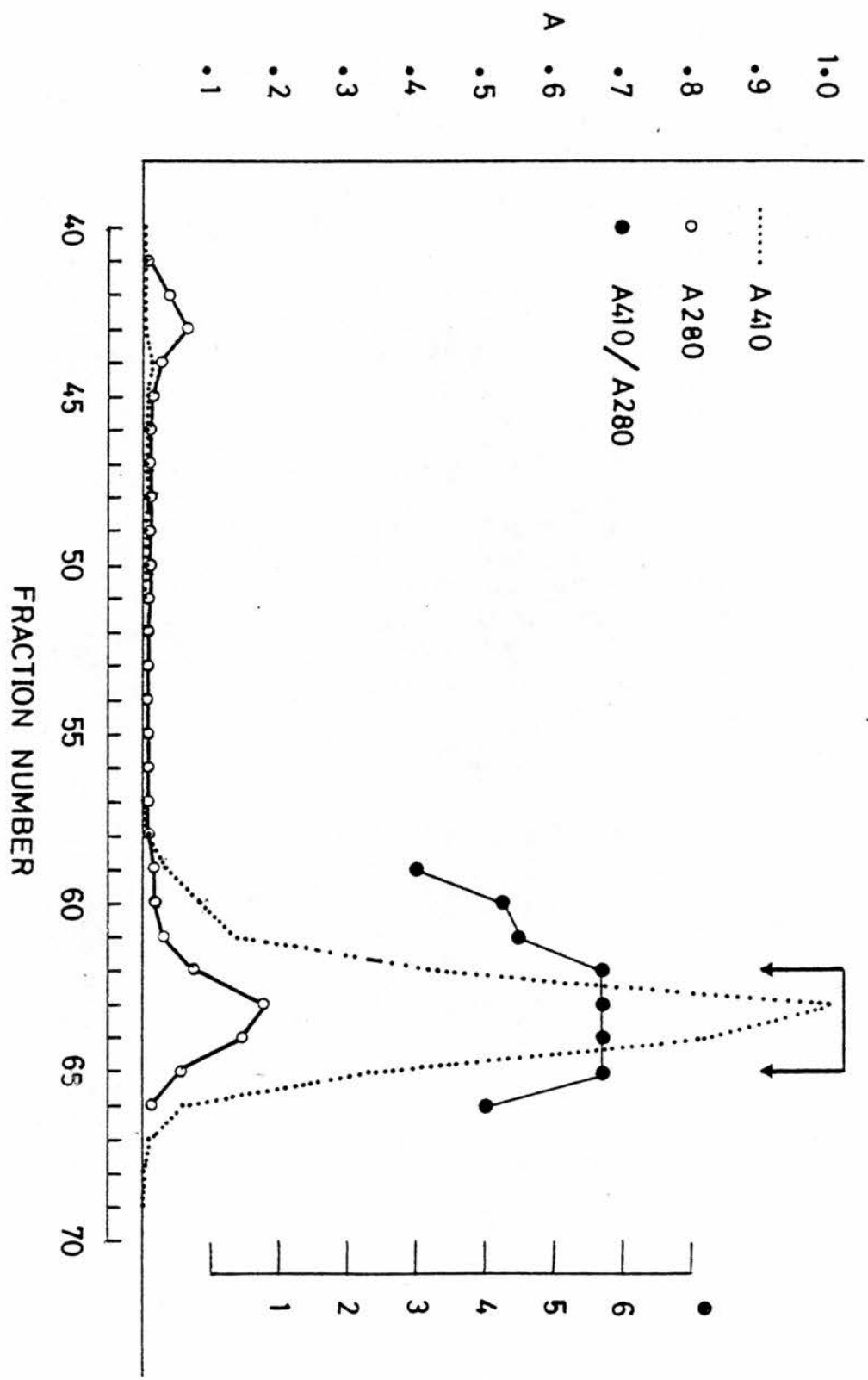


Fig. 24: CM-Cellulose Chromatography of Cytochrome c₅₅₃

Cytochrome c₅₅₃ was adsorbed to CM-cellulose (Whatman CM52) as described in text and eluted with a pH/ionic strength gradient of acetate buffer 10 mM (pH 4.5) - 100 mM (pH 5.6). Column size was 18 x 2.5 cm. The flow rate was 0.25 ml/minute. The arrows indicate fractions pooled for further treatment.

during purification of cytochromes (Pettigrew, G.W. personal communication), and have been postulated to be artifacts of the preparations and attributed to denaturation, polymerization and deamidation (Margoliash and Schejter 1966).

Typically 75-80% of the cytochrome applied to the CM-cellulose column was finally recovered (table 5) eluting in a sharp chromatographic peak (fig. 24).

iv. The Monitoring of Chromatographic Fractions

The ratio of the absorbance at either the α -peak in the reduced state or of the Soret peak in the oxidized state to the absorbance at 280 nm is usually used as a purity index during isolation procedures (Margoliash and Schejter 1966; Ambler 1963). Such a ratio is useful for monitoring the removal of contaminant proteins, but does not allow the assessment of the presence of modified forms of cytochromes (e.g. polymeric and deamidated forms). Such modified forms are usually identified by other means such as their carbon monoxide binding properties (Margoliash and Schejter 1966). Another problem in using this purity index is its limited usefulness when more than one 410 nm absorbing species are to be separated from each other. In such a case a misleading impression of the relative purity at the different stages of purification is obtained. Thus in the case of Campylobacter extracts all the cytochromes identified potentiometrically contribute to the absorption at 410 nm. These species are separated by the sephadex chromatography. Only after this stage is the 410 nm absorption due to a single species and can the ratio be used meaningfully as a purity index.

v. The Recovery of Cytochrome c₅₅₃

An estimate of cytochrome c₅₅₃ present in the cell extract can be made on the basis of the redox potentiometric data (table 4).

Approximately 18% of total cytochrome present in the cell extract represents cytochrome c_{553} . Accordingly the recovery of cytochrome c_{553} is 57% (table 5).

vi. The Concentration of CM-Cellulose Column Chromatographic Fractions

CM-cellulose column chromatographic fractions giving a ratio of A410/A280 nm of 5.8 were pooled. The pH at this stage was approximately 5.2 and was adjusted to 4.5 by titration with 1M acetic acid. Thus the concentration usually rose to approximately 100 mM Na acetate as measured by a conductivity meter. The cytochrome solution was then diluted to 5 times its volume with distilled water and adsorbed onto a small CM-cellulose column of 0.5-1 ml bed volume equilibrated with 10 mM sodium acetate buffer pH 4.5. The cytochrome was then eluted by the addition of 100 μ l aliquots of 100 mM sodium phosphate buffer pH 7.0 to the top of the column which was allowed to drain between additions. The cytochrome moved down the column in a sharp band of concentrated material which was recovered in a final volume of 0.5-1.5 ml.

vii. Desalting

Desalting of the cytochrome solution was required prior to amino acid analysis and dansylation. A sephadex G25 column 12 x 2 cm equilibrated with 0.1 mM sodium phosphate buffer pH 7.0/10 mM NaCl was used for desalting cytochrome c_{553} . The equilibration buffer was used for elution.

B. The Purity of Cytochrome c_{553}

Polyacrylamide gel electrophoresis, both in the native state (fig. 25) and under denaturing conditions (fig. 26), indicated that cytochrome c_{553} was obtained in an essentially homogeneous state. This is confirmed by other indices of purity namely:

(i) The ratio 410/280 across the chromatographic peak (fig. 24) is constant. This is indicative of the absence of contaminating proteins.

Table 5: Summary Purification of Campylobacter Cytochrome c₅₅₃

	Cells wt g	Vol mls	nmol Cytochrome	A ₄₁₀	A ₂₈₀	A ₄₁₀ / A ₂₈₀	%*	nmol** cyt c ₅₅₃	% Recovery cytochrome c ₅₅₃
Extract	10.8	36.5	1207	0.46	0.18	0.39	100	212	100
165,000 g Supernatant	-	34.5	343	0.43	1.12	0.38	28	-	-
Sephadex Fractions	-	500.0	150	0.03	0.006	5.00	12	-	-
CM-Cellulose Fractions	-	20.0	120	0.36	0.062	5.81	10	120	57

*Percentages were calculated relative to the total cytochrome present in the cell extract and not relative to cytochrome c₅₅₃ which is separated from other contaminating cytochromes only at the gel filtration level.

**nmol cytochrome c₅₅₃ were calculated from redox potentiometric data (table 4).

(ii) The minimum molecular weight as determined from the amino acid composition per haem (section D) is in good agreement with the molecular weight as determined by SDS (section F) and gel filtration (section F). This is a particularly good index of purity since any non-haem contaminating proteins would have led to a minimum molecular weight per haem higher than that obtained on SDS.

Also the fair agreement to integral values of mol amino acid per mol haem (table 8) and the presence of less than 0.1 mol arginine per mol haem (table 8) indicate that the preparation of cytochrome c_{553} is highly homogeneous.

(iii) Dansylation followed by hydrolysis yielded one major amino acid (section E) as the N-terminus.

(iv) The ratio of the absorbance at the α -peak to A_{280} can be used as a reliable index of purity (Margoliash and Walasek 1967). The ratios for a number of cytochromes are shown in table 6. The ratio for Campylobacter cytochrome c_{553} preparations, whose purity was unequivocally established as described above, is 1.08 (table 6). This relatively low ratio can be rationalized in terms of the lower absorbance at the α -peak (table 6). Low α/A_{280} values have been reported for a number of protozoan cytochromes and attributed to decreased α -peak extinction (Pettigrew 1979). Hence in addition to indices of purity described above the value of $A_{553 \text{ nm}}/A_{280 \text{ nm}}$ was taken as a reliable and confirmatory index of purity of cytochrome c_{553} during subsequent preparations.

C. The Spectral Properties of Cytochrome c_{553}

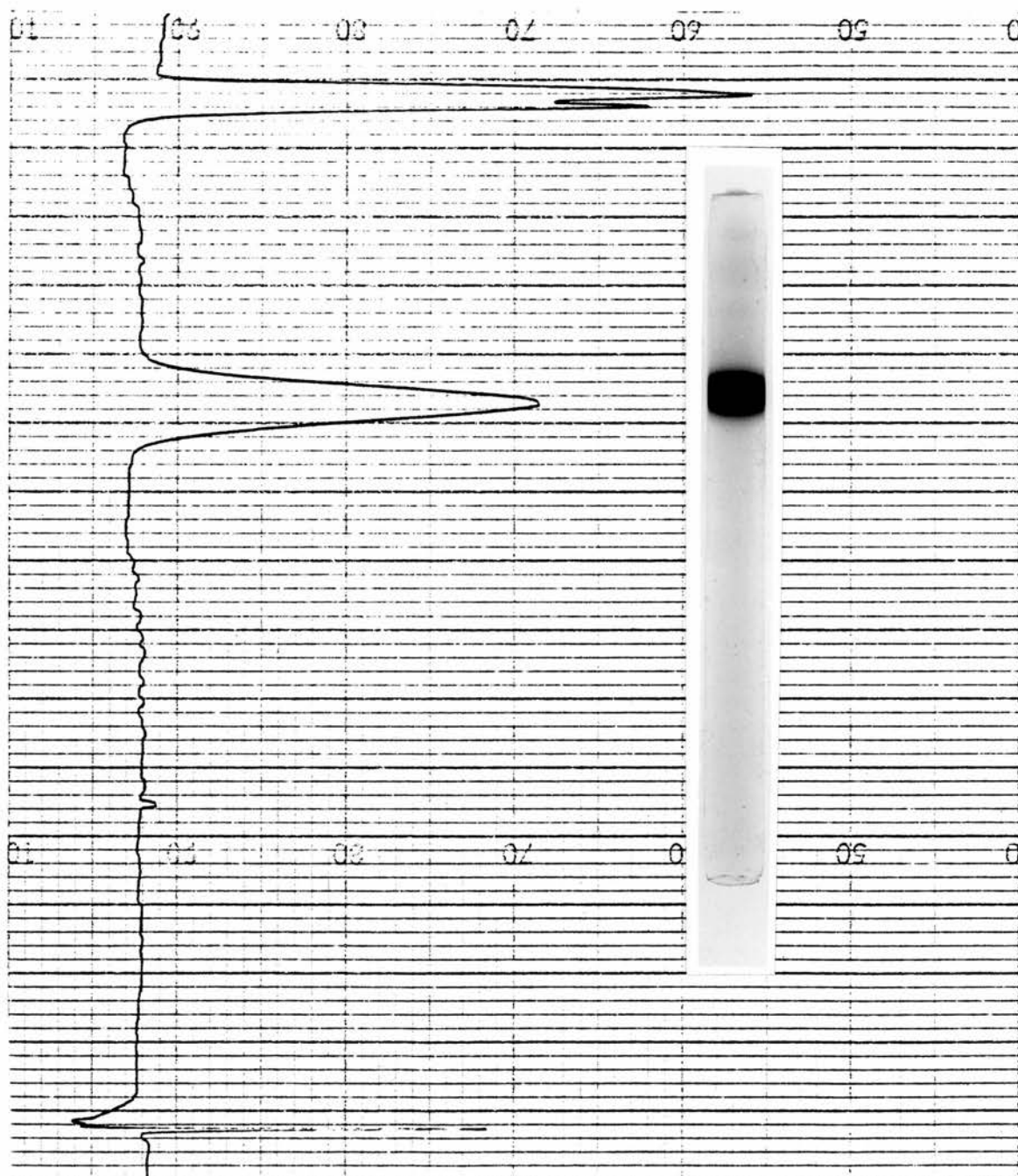
For maximum reproducibility the same 100 μ l capacity glass micropipette was used for dispensing 3 aliquots from the same solution of cytochrome. The 3 aliquots were used for the pyridine haemochrome

Table 6.

<u>Cytochrome</u>	<u>$\epsilon_{\text{mM } \alpha\text{-peak}}$</u>	<u>α/A_{280}</u>
1. Horse Heart	27.7	1.2
2. <u>Campylobacter</u> c_{553}	19.75	1.08
3. <u>Crithidia</u> c_{557}	24.70	1.02
4. <u>Pseudomonas</u> c_{551}	30.0	1.25

1. Margoliash and Frohwirt (1959)
2. This Thesis
3. Pettigrew 1979
4. Ambler 1963

Fig. 25: Native Gel Electrophoresis of Purified Campylobacter
Cytochrome c₅₅₃



3 nmol of purified cytochrome c₅₅₃ electrophoresed on 10% gel.
Gel preparation and electrophoretic conditions were described in
methods. The gel was scanned at 410 nm before being stained for protein.

Fig. 26: SDS Gel Electrophoresis of Cytochrome c₅₅₃



SDS electrophoresis on 3 nmol cytochrome c₅₅₃ was carried out essentially as described by Weber and Osborne 1969.

Electrophoretic conditions and staining of gels were carried out as described under methods.

spectrum, the oxidized and reduced spectrum and for amino acid analysis.

i. The Pyridine Haemochrome Spectrum

The pyridine haemochrome spectrum of the cytochrome is shown in fig. 27 and exhibits the α -peak absorption maximum at 550 nm typical of c-type cytochromes (ϵ_{mM} 31.18 Bartsch 1971).

ii. The Native Spectrum

The spectrum of Campylobacter cytochrome c_{553} is shown in fig. 28. Table 7 lists the absorption maxima, the mM extinction coefficients and the absorbance ratios. The extinction coefficients were calculated from the concentration of cytochrome in terms of haem as determined in (i) above.

The α -peak (553 nm) is prominently asymmetric with a shoulder at 548 nm. The α/β ratio (1.27) is low and the $\gamma_{\text{red}}/\alpha$ ratio (7.69) is high as compared to mitochondrial cytochrome c where the respective ratios are 1.9 and 5.0 (Margoliash and Walasek 1967; Lemberg and Barrett 1973). The ferricytochrome shows a peak at 695 nm (fig. 29) indicative of coordination of a methionine residue to the haem iron and a low spin character; but also exhibits a small amount of a high spin component as indicated by an absorption shoulder at 620 nm.

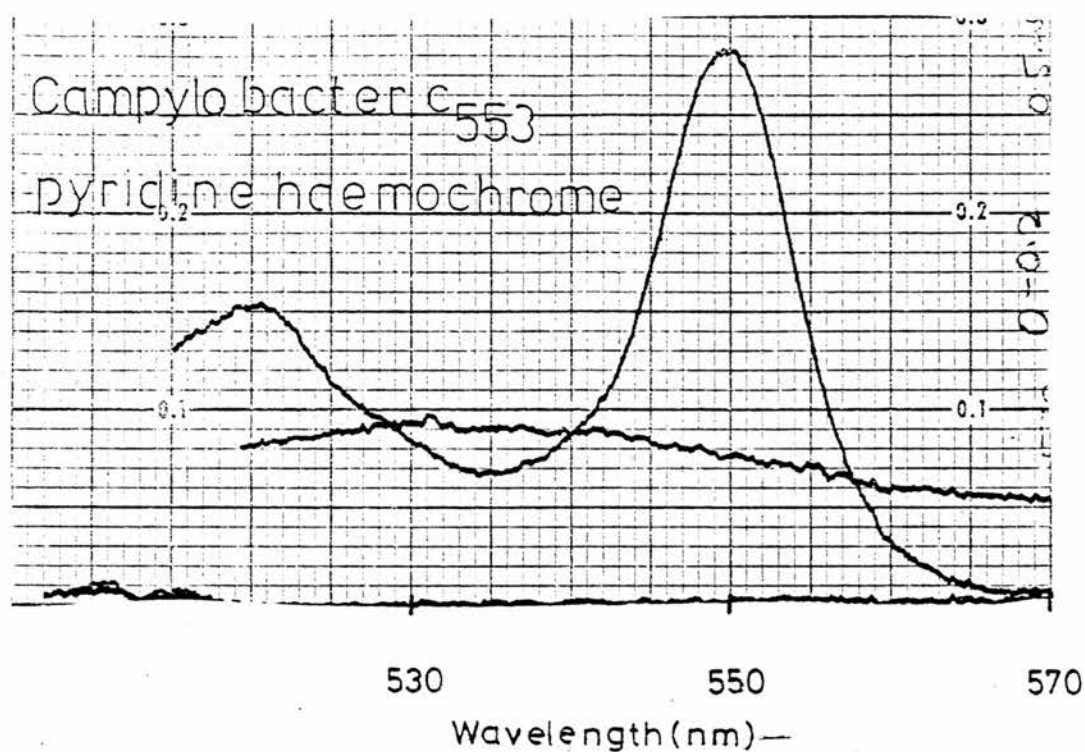
The spectrum of mitochondrial cytochrome c as well as the expanded α and β regions are shown in fig. 30 and 31 for comparison. Fig. 31 also includes the α and β peaks of Chlorobium cytochrome c_{555} , another cytochrome with an asymmetric α peak. Full comparison of cytochrome c_{553} with similar cytochromes will be presented in the final chapter.

D. The Amino Acid Composition of Cytochrome c_{553}

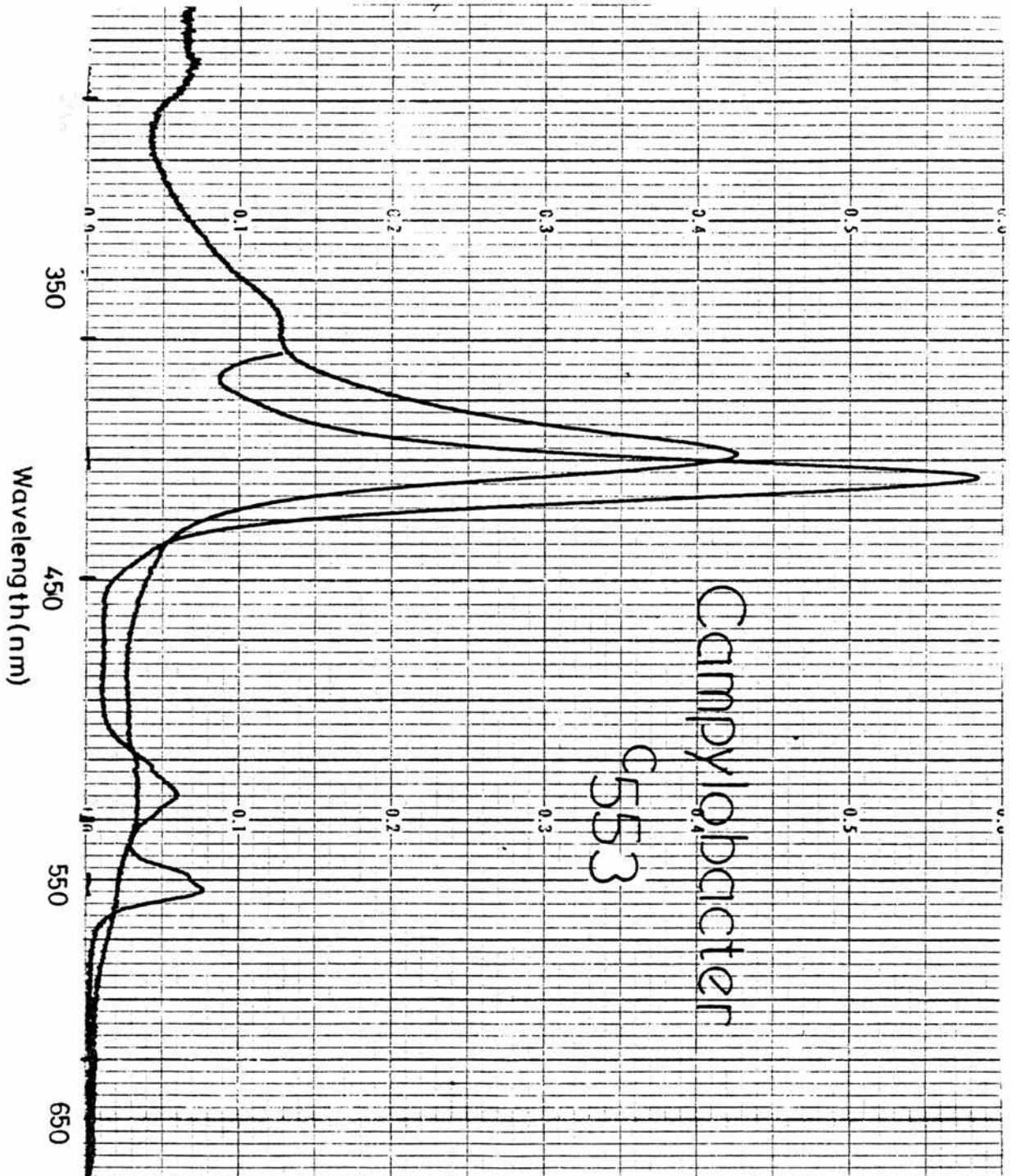
i. Amino Acid Analysis

The result of amino acid analysis of cytochrome protein, corresponding to 11.55 nmol haem determined by the pyridine haemochrome

Fig. 27: Pyridine Haemochrome Cytochrome c₅₅₃



The pyridine haemochrome spectrum of *Campylobacter c₅₅₃* was determined as described in materials and methods. The spectrum was recorded in a Cary model (219) spectrophotometer.

Fig. 28: The Absorption Spectrum of Cytochrome c_{553} 

The absorption spectrum of cytochrome c_{553} in 0.1M sodium phosphate buffer pH 7.0 was recorded on a Cary model 219 spectrophotometer.

Fig. 29: Absorption Spectrum of Cytochrome c_{553} in the Oxidized State (510 nm - 750 nm)

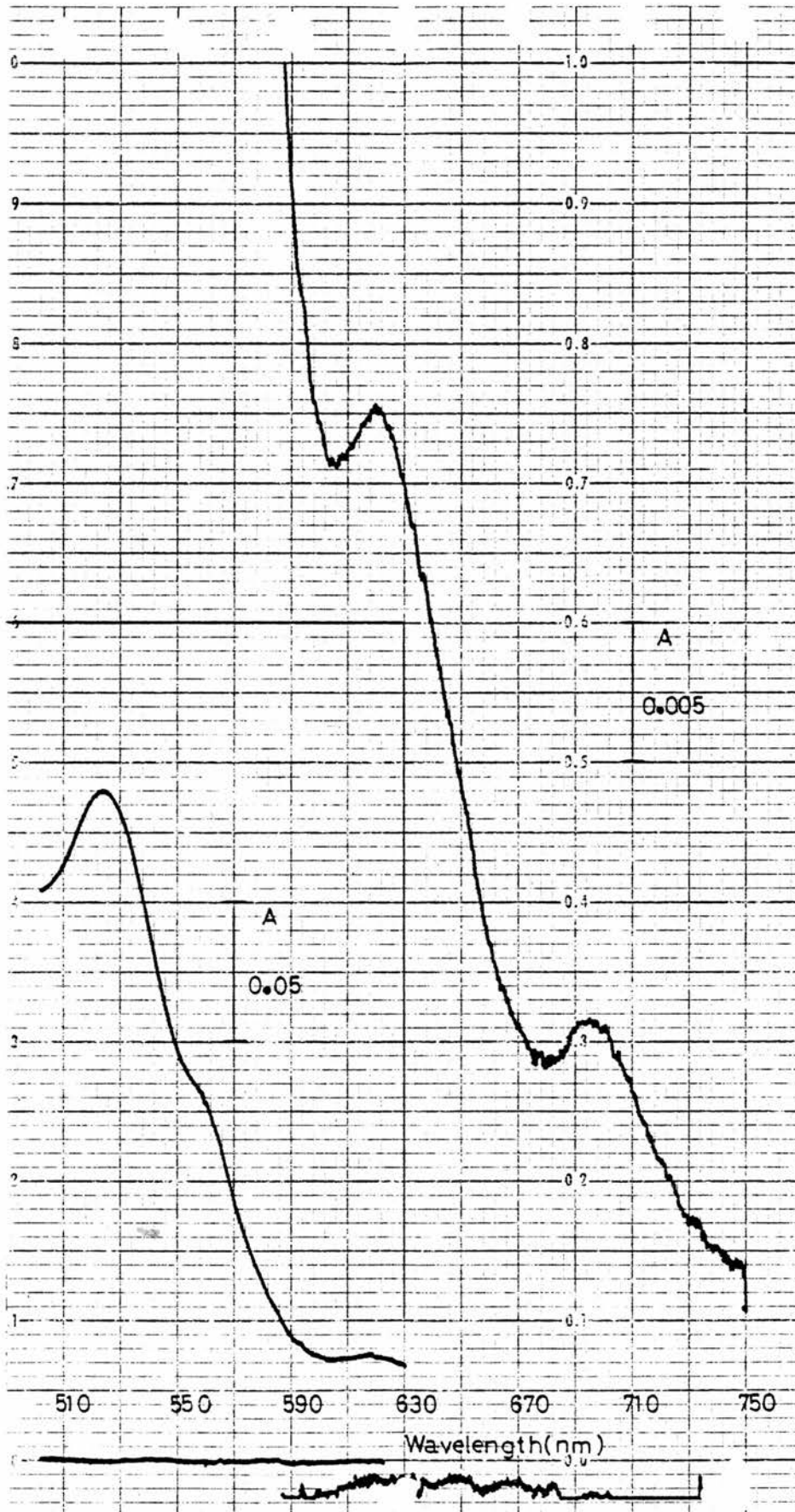


Table 7: Features of the Absorption Spectra of CampylobacterCytochrome c₅₅₃Absorption MaximaFerricytochrome

Soret 408 nm

524 nm

695 nm

Ferrocyclochrome

416 nm

 β 522 nm α 553
(548) nmmM Extinction CoefficientsWavelengths ϵ (mM⁻¹cm⁻¹)Absorbance Ratios

408 (ox)

110.2

416 red/553 red 7.69

416 (red)

151.8

553 (red-ox)

15.59

553 red/522 red 1.27

553 (red)

19.95

522 (red-ox)

7.28

522 (red)

15.59

280 (ox)

18.17

553 red/280 ox 1.09

524 (ox)

8.31

695 (ox)

0.56

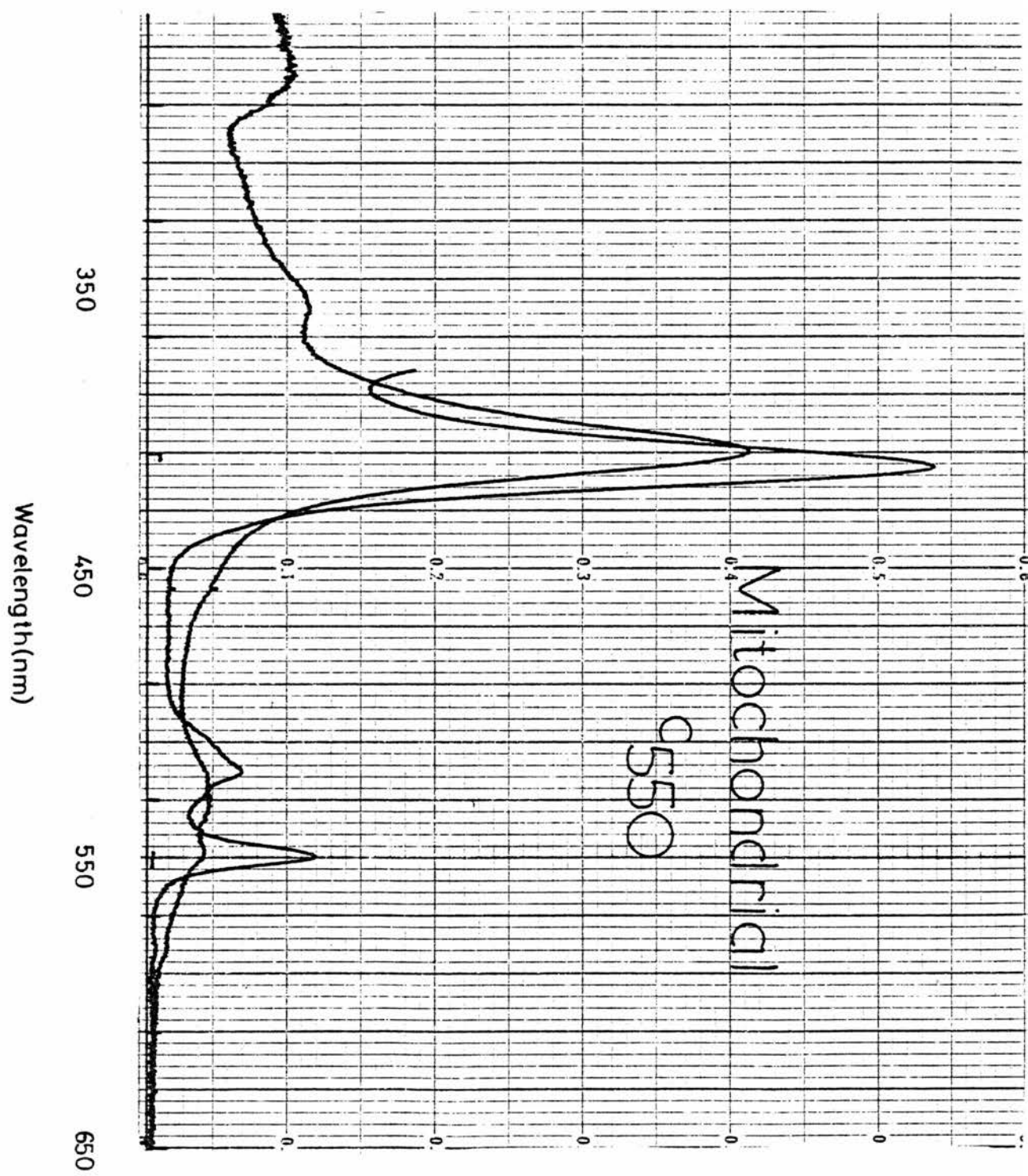
Fig. 30: Absorption Spectrum Mitochondrial Cytochrome c

Fig. 31: Expanded α -Peaks of Cytochrome c_{553} , Chlorobium c_{555}
and Mitochondrial Cytochrome c

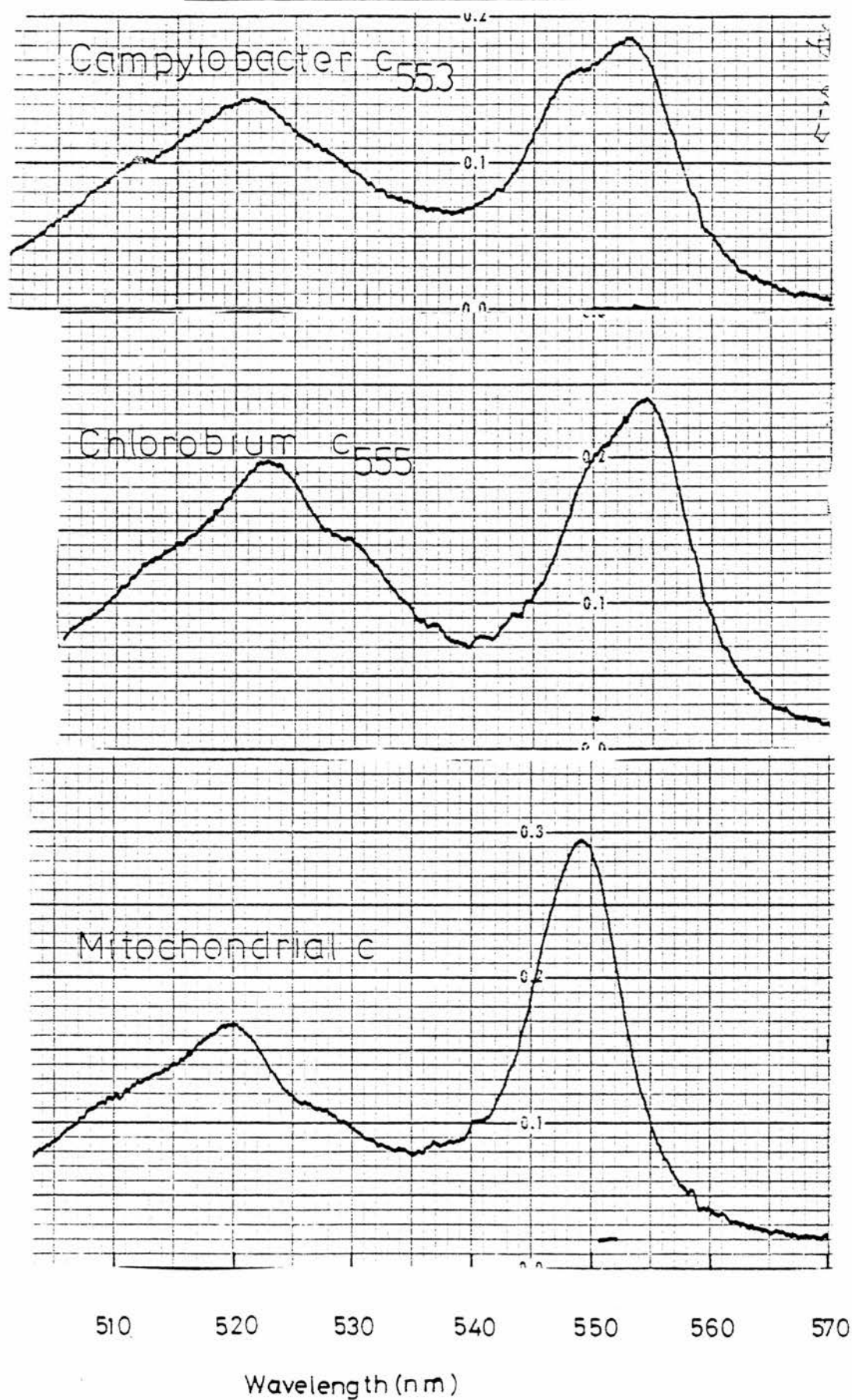


Table 8: Amino Acid Analysis*

Amino acids	nmol		Residue per mol haem	mol/mol haem Integral Values
	20 hours	70 hours		
Asp	137.07	130.13	11.57	12
Thr	44.78	41.31	3.99	4
Ser	21.63	18.75	1.97	2
Glu	33.31	31.83	2.82	3
Pro	54.39	57.68	4.85	5
Gly	66.52	64.20	5.66	6
Ala	137.08	125.30	11.38	11
Cys	-	-	-	2**
Val	41.09	45.32	3.93	4
Met	44.96	44.0	3.85	4
Ile	42.30	48.10	4.17	4
Leu	42.05	42.05	3.64	4
Tyr	42.30	38.87	3.51	4
Phe	11.02	12.96	1.03	1
His	13.88	11.52	1.10	1
Lys	103.02	100.49	8.80	9
Arg	1.02	1.12	0.09	0

*11.55 nmol haem per sample - as determined by the pyridine haemochrome method - were taken for analysis.

**Assumed (see text).

Total residues per molecule 76.

Minimum molecular weight (apoprotein + haem) 8713.

Table 8: Amino Acid Analysis

In calculation of the residues/mol haem the 70 hours hydrolysis figures in nmol for valine and isoleucine were utilized. The figures are higher than the 20 hours hydrolysis figures and are hence more representative of recovery. For serine and threonine - extrapolation of the 20 hours and 70 hours hydrolysis figures to zero time was necessary before the ratio residue/mol haem could be calculated. For all other amino acids the averages of the 20 hours and 70 hours hydrolysis figures were used in the calculation.

method, is shown in table 8. In such analysis consideration has to be given to the fact that some peptide bonds require longer times for optimum hydrolysis while some amino acids are destroyed by such treatment. Thus bonds involving isoleucine and valine often require more than 40 hours of hydrolysis while serine and threonine are partly destroyed by 20 hours and progressively lost beyond 20 hours. Cysteine and tyrosine can be partly destroyed by acid hydrolysis though both are protected by the presence of thioglycollic acid. Tryptophan is markedly labile and almost none survives. Amino acid analysis has been reviewed by Blackburn 1968 and Gardner 1981.

ii. Cysteine Content

No cysteine was detected on the chromatogram. Thioether bonds are presumably not hydrolysed by acid and the two cysteines bonded to haem are consequently lost during amino acid analysis. Cysteine is a difficult amino acid to measure, the most accurate results being obtained by oxidizing it to cysteic acid (Ambler 1981). This would entail removal of the haem which can be achieved by treatment of the cytochrome with HgCl_2 in 8M urea 0.1M HCl at 37°C for 20 hours followed by gel filtration (Ambler and Wynn 1973). It was felt that since treatment of the protein with mercuric chloride followed by subsequent treatment utilizes greater amounts of protein than that usually used for amino acid analysis, the cost in terms of precious cytochrome was not justified. In general however there are two cysteine residues per haem in cytochrome c; the exception being those cytochromes c_5 of the Pseudomonads which contain an extra pair of cysteine residues (Ambler 1973), some protozoan cytochromes containing one cysteinyl residue (Pettigrew 1979), and yeast cytochrome c which contains 3 cysteinyl residues (Lemberg and Barrett 1973). In view of this, cytochrome c_{553} may contain only the two cysteinyl residues engaged in haem attachment.

iii. Estimation of Tryptophan

Since tryptophan is very susceptible to destruction by hydrochloric acid hydrolysis, no tryptophanyl residues were identified during amino acid analysis (although good recoveries of this amino acid might have been attained after hydrolysis with mercaptoethane sulphonic acid).

The absorption of proteins at 280 nm is due mainly to their content of tryptophan and tyrosine and in cytochromes the haem contributes to absorption in that region. Thus the tryptophan content of a cytochrome can be estimated provided certain assumptions are made:

- a) the extinction coefficients of both protein tyrosine and protein tryptophan at 280 nm as such do not significantly differ from their extinction coefficients in the free state;
- b) the haem contribution to the extinction at 280 nm can be calculated for well-studied cytochromes by subtracting the contribution of both tryptophan and tyrosine from the total absorption at that wavelength.

An estimate of tryptophan in Campylobacter cytochrome c_{553} by such means is shown in table 9. The calculations indicate complete absence of tryptophan in the cytochrome.

E. The N-Terminus and N-Terminal Sequence of Cytochrome c_{553}

Amino terminus determination is the first step in sequence analysis and is also a test of purity. The presence of more than one residue may be due to an impurity or the occurrence of "raggedness". Dansylation of the amino terminus followed by hydrolysis of the peptide and identification of the dansylated amino acid(s) is a highly sensitive index of these possibilities.

The dansylation, hydrolysis and chromatography of the dansyl amino acids of Campylobacter cytochrome c_{553} was carried out as described under materials and methods. Figures 32 and 33 show chromatography of

Table 9: Estimation of Tryptophan in Campylobacter Cytochrome c₅₅₃

	Horse heart cytochrome c	Euglena cytochrome c ₅₅₈	Campylobacter cytochrome c ₅₅₃
	$\epsilon_{mM_{280}} = 23.2^*$	$\epsilon_{mM_{280}} = 30.3^{**}$	$\epsilon_{mM_{280}} = 18.17^+$
Tryptophan ($\epsilon_{mM_{279}} = 5.2^{++}$)	1^*	2^{**}	$x = 0$
Tyrosine ($\epsilon_{mM_{278}} = 1.1^{++}$)	4^*	5^{**}	4^+
Contribution of Tryptophan to A ₂₈₀	$5.2 \times 1 = 5.2$	$5.2 \times 2 = 10.40$	$5.2 \times x = ?$
Contribution of tyrosine to A ₂₈₀	$1.1 \times 4 = 4.4$	$1.1 \times 5 = 5.50$	$1.1 \times 4 = 4.4$
Total contribution of Tryptophan and tyrosine to A ₂₈₀	$(5.2 + 4.4) = 9.6$	$(10.4 + 5.5) = 15.90$	
Deduced haem contribution to A ₂₈₀	$(23.2 - 9.60) = 13.6$	$(30 - 15.90) = 14.10$	
Average contribution of haem to A ₂₈₀	$\frac{(13.6 + 14.1)}{2} = 13.85$		

Estimated number of Tryptophan residues

0

References:

*Margoliash et al. 1959; **Pettigrew 1973; ⁺This Thesis; ⁺⁺Mahler and Cordes 1966;
^{*}Margoliash et al. 1962.

standard DNS amino acid mixture on one face of a polyamide sheet and that of the dansylated and hydrolyzed peptide on the other face. In both cases DNS NH_2 was included as a marker.

Comparison of the positions of the spots identified under U.V. light unequivocally establishes alanine as the N-terminal amino acid of Campylobacter cytochrome c_{553} . The presence of O-DNS Tyr and ϵ -DNS Lys is expected from side chain dansylation reactions. The presence of extra spots of much lower intensity is a qualitative reflection on the purity of the preparation. This together with electrophoretic studies and amino acid analysis confirm that the preparation is essentially homogeneous.

The amino acid terminus of Campylobacter cytochrome c_{553} was also confirmed and extended by sequence analysis of the N-terminus (fig. 34) which gives the sequence:

ala-asg-gly-ala.

The identification of only 4 amino acid residues might be explained in terms of the small amount of protein (63 nmol cytochrome) used for sequencing, a lower repetitive yield or loss of protein during sequencing, or a combination of these.

F. The Molecular Weight of Cytochrome c_{553}

i. Molecular Weight as Determined by Sephadex Chromatography

A molecular weight for cytochrome c_{553} was determined by chromatography on a sephadex G75 column. Column calibration and experimental details are given in fig. 35. The chromatographic behaviour of cytochrome c_{553} was consistent with a molecular weight of 11,000.

Fig. 32: Chromatography of Standard Dansyl Amino Acids on
Polyamide Sheets

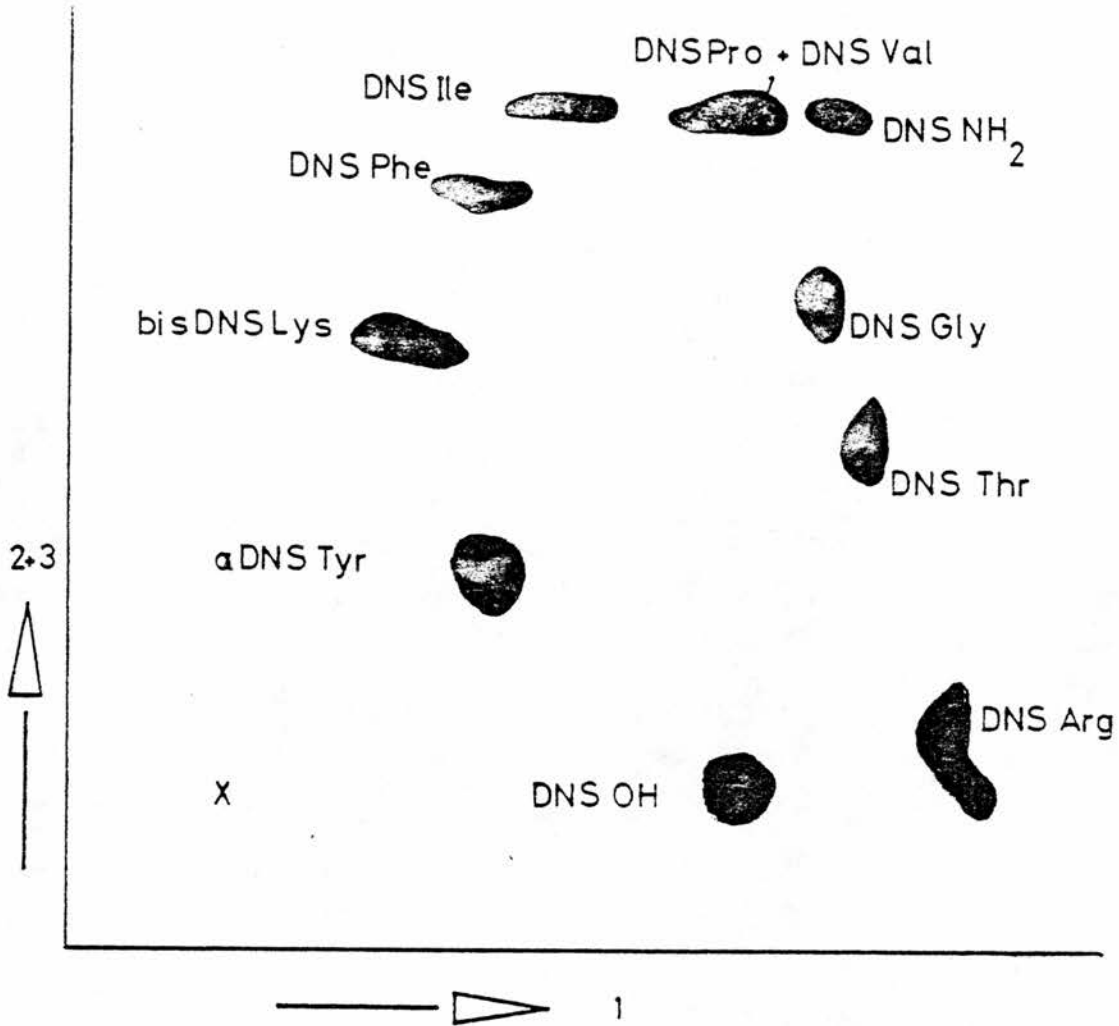
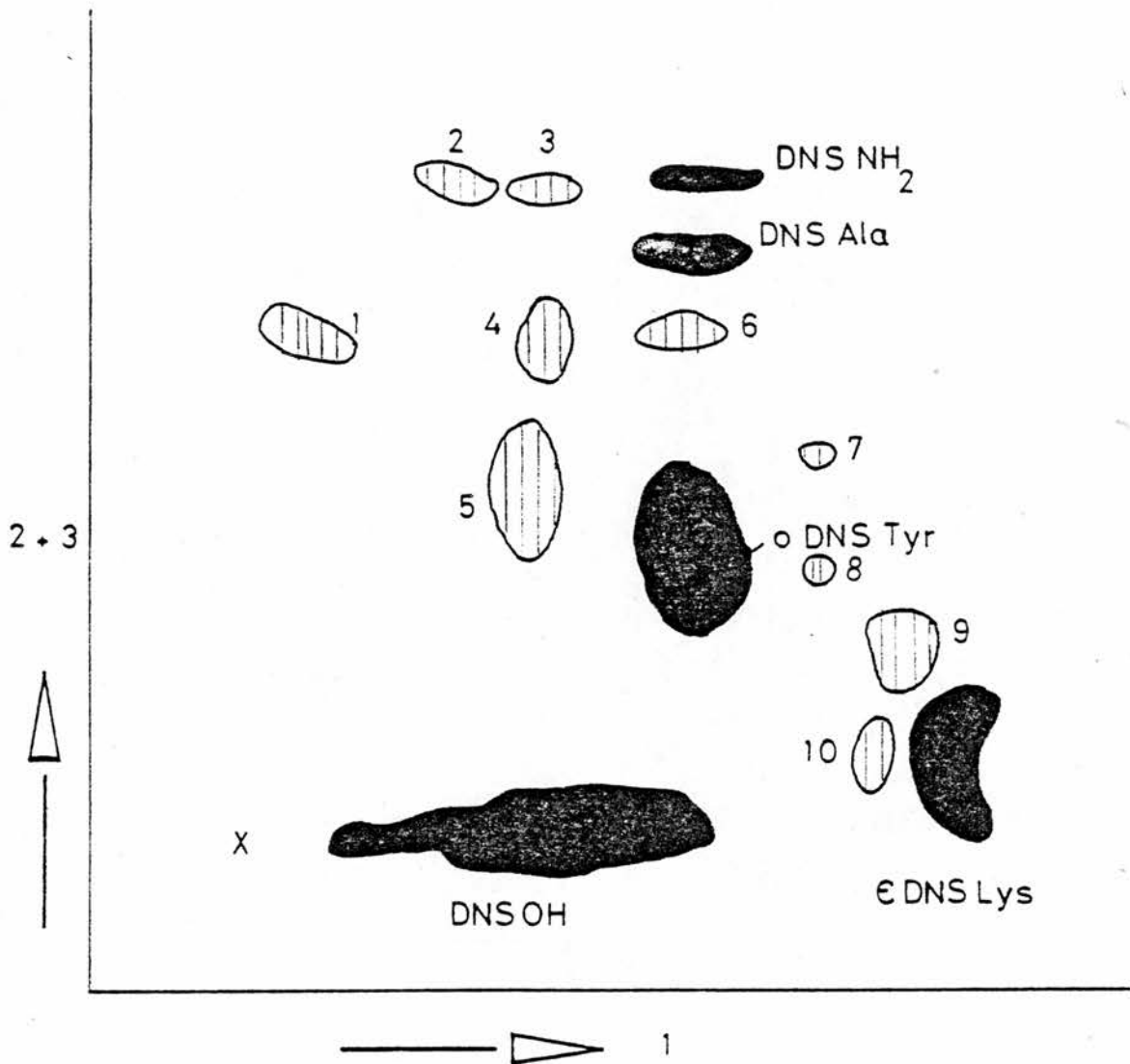


Fig. 33: Chromatography of Dansylated and Hydrolyzed Cytochrome c₅₅₃
on Polyamide Sheets



Key:

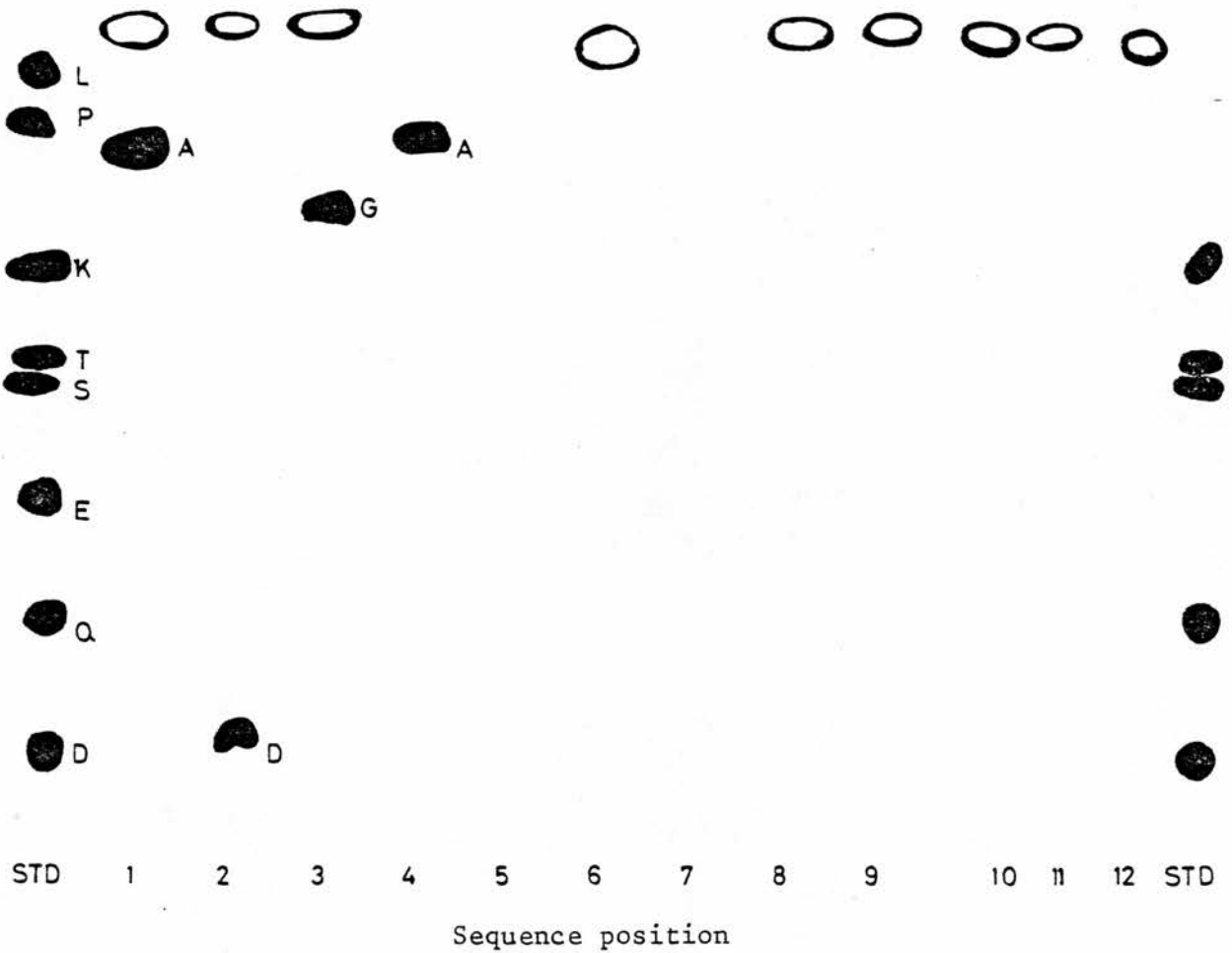
- | | |
|------------------|-----------------|
| 1. DNS - Phe | 6. DNS - Gly |
| 2. DNS - Ile | 7. DNS - Thr |
| 3. DNS - Val | 8. DNS - Ser |
| 4. DNS - O - Tyr | 9. DNS - - Lys |
| 5. DNS - O - Tyr | 10. DNS - - Lys |

Black spots represent approximately 10 times the intensity of the cross hatched spots.

Fig. 33: Chromatography of Dansylated and Hydrolyzed Cytochrome c₅₅₃
on Polyamide Sheets

Dansylation, hydrolysis and chromatography of *Campylobacter* cytochrome c₅₅₃ was carried out as described under materials and methods.

α -DNS histidine and DNS arginine have mobilities very similar to that of ϵ -DNS lysine; however these DNS amino acids can be excluded on the basis of the amino acid analysis which shows the absence of arginine and the presence of only one histidyl group which by analogy with all other c-type cytochromes will occur immediately after the haem attachment site and form the 5th iron ligand.

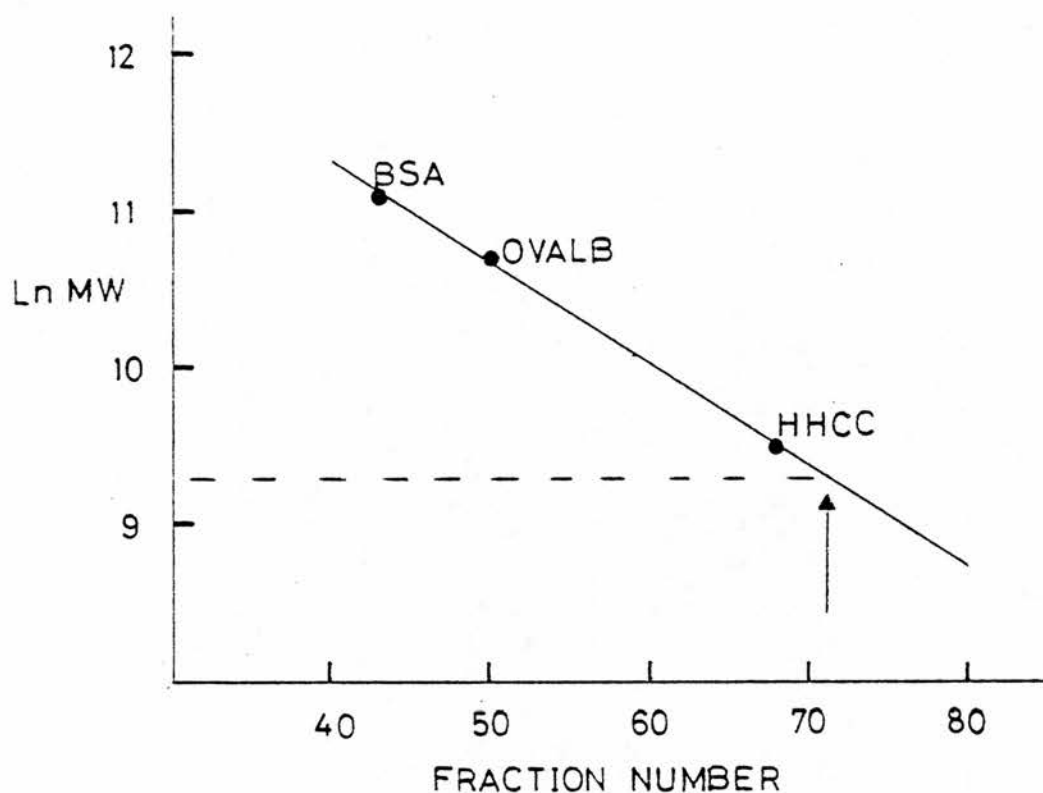
Fig. 34: Sequence Determination Campylobacter Cytochrome c₅₅₃Chromatographic Separation of PTH Amino Acids

Key:

STD = Standards, A = Ala (pink), D = Asp (red), E = Glu (green),
 G = Gly (orange), K = Lys (colourless), L = Leu (colourless),
 P = Pro (pink), Q = Gln (green), S = Ser (red), T = Thr (yellow).

Fig. 34: Sequence Determination Campylobacter Cytochrome c₅₅₃

N-terminal sequence analysis was carried out on 63 nmol cytochrome c₅₅₃. Experimental details are given in materials and methods (chapter II). Spots were identified by their fluorescence under ultraviolet light. Many PTH aminoacids can be identified by their colour after reaction with ninhydrin as well as by their mobility. The colourless spots (○) are by-products of the Edman reaction. PTH aminoacids of similar mobility such as leucine and phenylalanine which do not react with ninhydrin and therefore remain colourless can be identified after back hydrolysis. This however could not be done because of lack of material.

Fig. 35: Molecular Weight Cytochrome c₅₅₃ on Sephadex G75

Arrow indicates peak chromatographic fraction (cytochrome c₅₅₃).

HHCC = horse heart cytochrome c

BSA = bovine serum albumin

OVALB = ovalbumin.

The molecular weight of cytochrome c₅₅₃ was determined in Sephadex G75 column of 90 x 3.5 cm dimensions. The void volume of the column was determined using blue dextran (molecular weight 2,000,000) and the column calibrated with bovine serum albumin, ovalbumin and horse heart cytochrome c. 25 mM Tris HCl pH 8.0/100 mM NaCl was used for equilibration and elution. Fractions of 7 ml each were collected. The peak cytochrome c₅₅₃ fraction corresponds to a molecular weight of 11,000.

ii. Molecular Weight as Determined by SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis of cytochrome c_{553} was carried out as described in fig. 26. The relative mobility of cytochrome c_{553} to horse heart cytochrome c is shown in fig. 36; and that of molecular weight markers in fig. 37. The relative mobility of cytochrome c_{553} in SDS gels corresponds to a molecular weight of 8,500 (fig. 38).

iii. Molecular Weight from Amino Acid Analysis

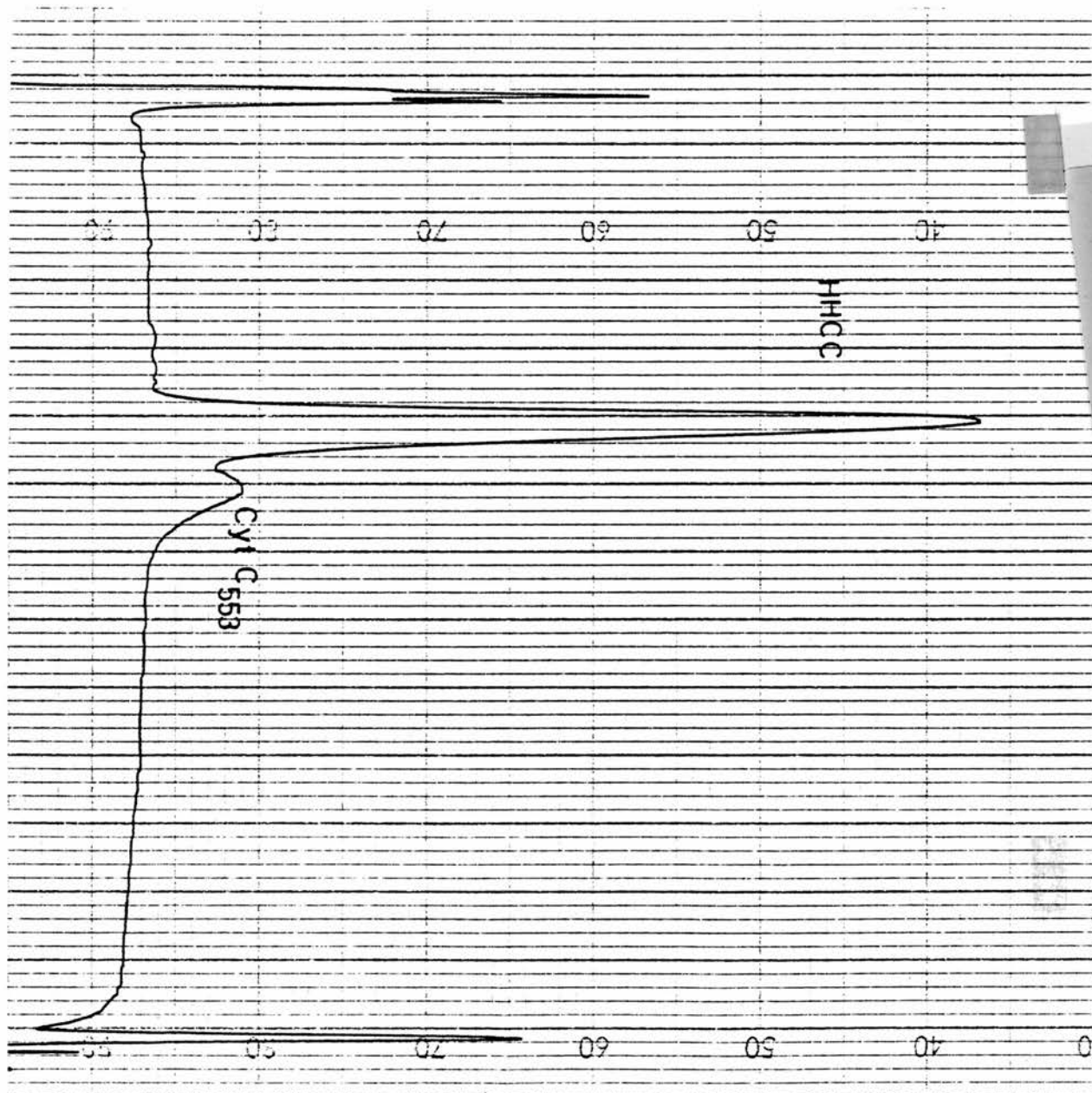
The minimum molecular weight of cytochrome c_{553} as determined by amino acid analysis and haem content is 8713 (table 8). The correspondence of this to that determined by SDS gel electrophoresis has already been discussed as an index of purity. This correspondence also indicates the monohaem nature of the cytochrome. Comparison of these molecular weights to that obtained on sephadex shows that cytochrome c_{553} is isolated as a monomer.

G. The Midpoint Potential of Purified Cytochrome c_{553}

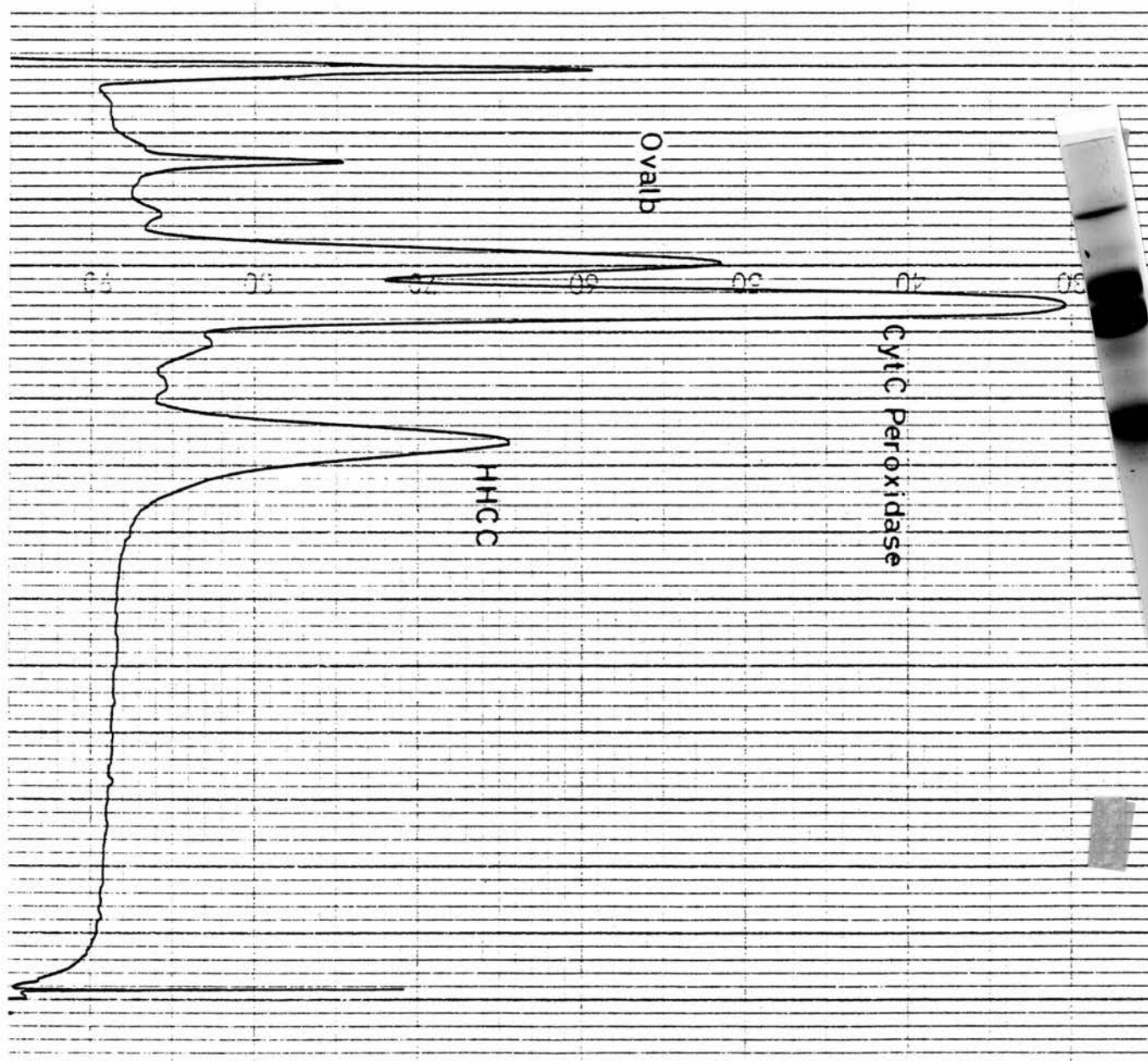
The method of mixtures (Wilson 1978) was chosen for determination of the midpoint potential of the purified Campylobacter cytochrome c_{553} . The couple Fe^{2+}/Fe^{3+} EDTA was used since at pH 7.0 its midpoint potential is close to the expected midpoint potential of the cytochrome as postulated from redox titrations of the crude fractions (chapter III). The theory and experimental details of the method of mixtures have been described under materials and methods (chapter II).

The spectra of the titration of the cytochrome are shown in fig. 39, and exhibit an asymmetric α -peak at 553 nm throughout. The midpoint potential was determined after titration from:

a) the plot for the method of mixtures (fig. 40). The value obtained is +103 mV.

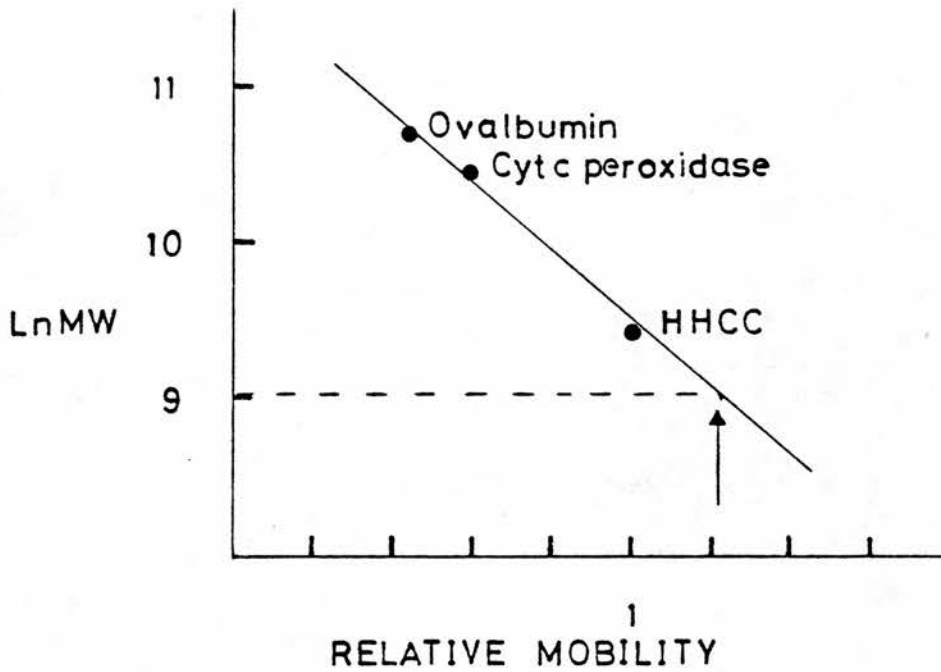
Fig. 36: SDS Electrophoresis of Cytochrome c₅₅₃ and Horse HeartCytochrome c

3 nmol horse heart cytochrome c and 1 nmol cytochrome c₅₅₃ were electrophoresed in 10% SDS gels. Preparation of the gels and electrophoretic conditions are described under materials and methods. The gel scan is for the 410 nm absorbance, the photograph for the gel after staining for protein.

Fig. 37: SDS Electrophoresis of Marker Proteins

1 nmol each of horse heart cytochrome c ovalbumin and cytochrome c peroxidase were electrophoresed in 10% gels. Preparation of the gels and electrophoretic conditions were described under materials and methods. The gel scan is for the absorbance at 560 nm after staining for protein.

Fig. 38: Molecular Weight Determination of Campylobacter
Cytochrome c_{553} by SDS Electrophoresis



The arrow indicates the relative mobility of cytochrome c_{553} which corresponds on a molecular weight of 8500 relative to horse heart cytochrome c (HHCC).

Fig. 39: The Spectra of Titration of Cytochrome c_{553} with Ferrous Ammonium Sulphate (Method of Mixtures)

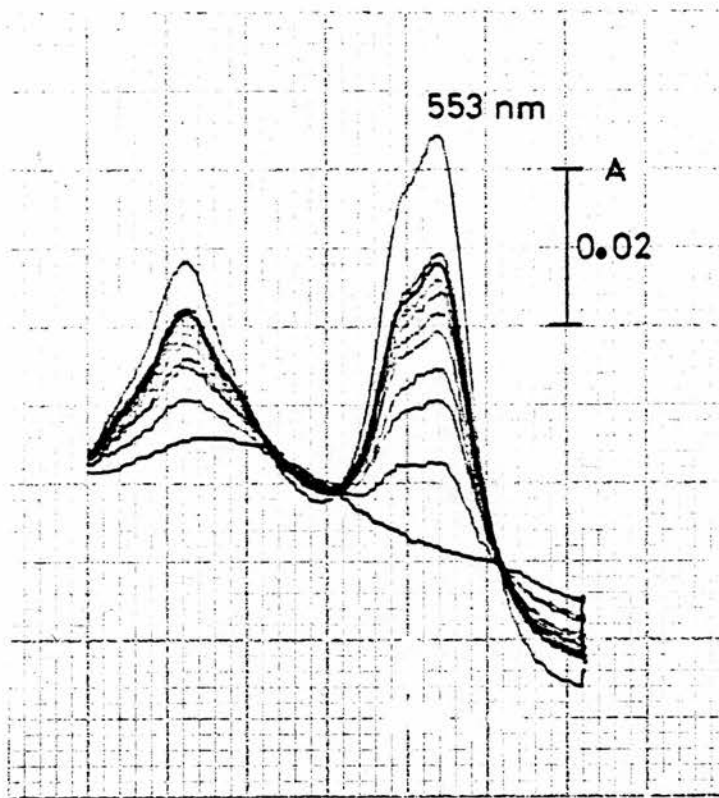
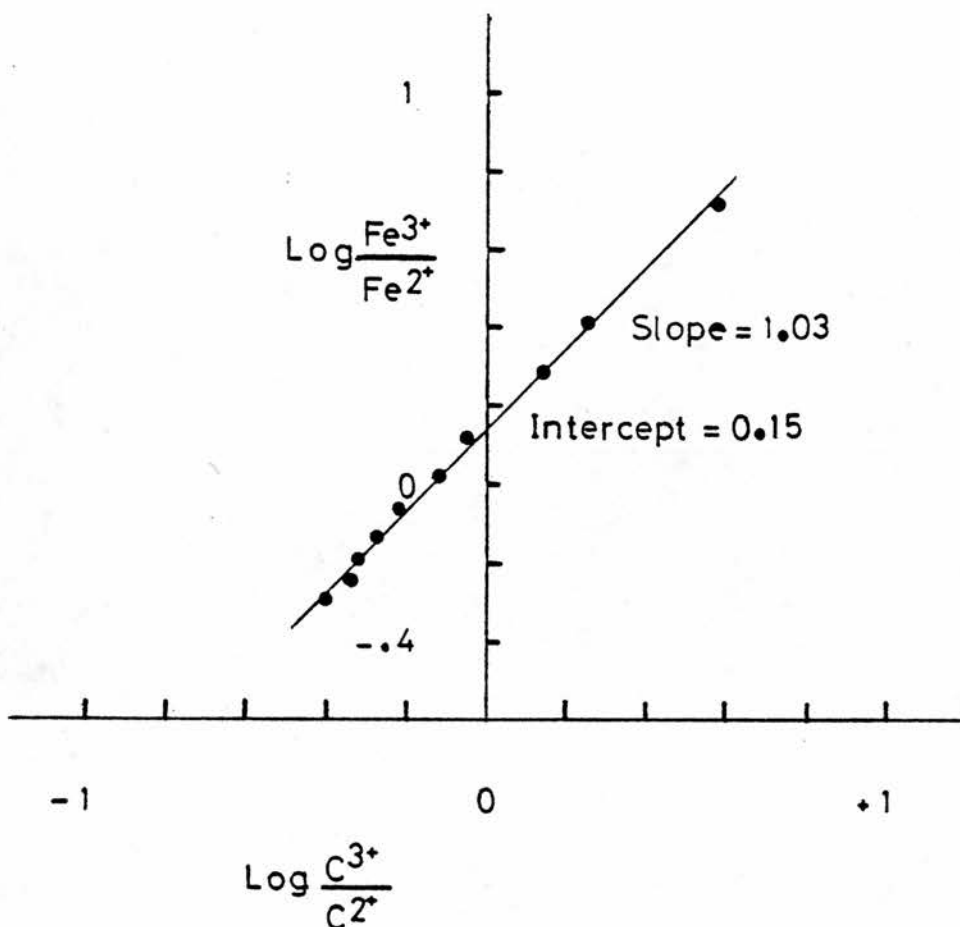


Fig. 40: Redox Potential of Campylobacter Cytochrome c₅₅₃
(Method of Mixtures)



Titration of the cytochrome in presence of 0.1 mM Fe^{3+} /10 mM EDTA was carried out by μl additions of 100 mM ferrous ammonium sulphate. Experimental details are given in methods. The line represents the best fit to the experimental points. The midpoint potential of cytochrome c₅₅₃ was calculated as follows:

$$\begin{aligned}
 E_{m_{c_{553}}} &= E_{m_{\text{Titrant}}} + 0.06 \times \text{Intercept} \\
 &= 0.094 + 0.06 \times 0.15 \\
 &= 0.103 \text{ V.}
 \end{aligned}$$

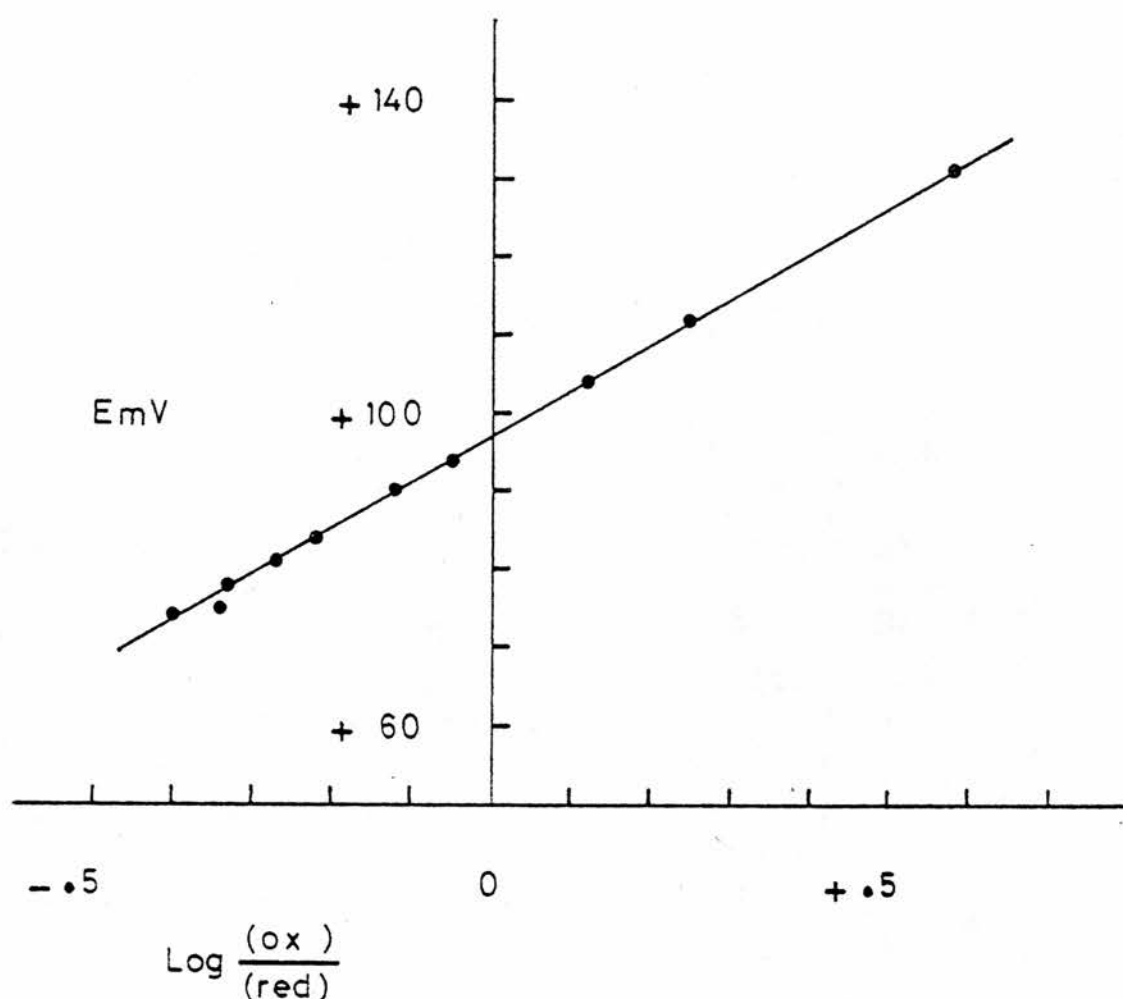
b) The plot of the observed potentials vs $\log (\text{ox})/(\text{red})$ cytochrome fig. 41. The value obtained is +97 mV. The good agreement of the experimental points with a 60 mV slope line is in contrast to the potentiometric titrations of the crude fractions in chapter III. This together with the spectra of the titration (fig. 39) are indicative of the presence of a single haem species. It is noteworthy that the values obtained for the midpoint potential of the purified cytochrome c_{553} are in good agreement with each other and with the values obtained on titration of the crude cell extract and 100,000 g supernatant (table 4).

H. Conclusions and Discussion

The objectives outlined at the beginning of chapter IV have been realized and the results presented are unequivocal evidence for the validity of proposals put forward in chapter III with respect to the presence and characteristic features of cytochrome c_{553} . The cytochrome can be described as:

- (i) of small molecular weight (8713)
- (ii) of positive midpoint potential (+103 mV)
- (iii) of probable acidic nature as evidenced by elution from CM-cellulose at pH 5.2
- (iv) of low spin and possessing an asymmetric α -peak at 553 nm with a shoulder at 548 nm.

The cytochrome clearly falls in the group of low spin high potential cytochromes c (chapter I). Numerous similar cytochromes c with positive midpoint potentials, low spin and small molecular weight have been isolated from bacterial sources. A number of these exhibit asymmetric α -peaks. Cytochrome c_{553} will be the subject of comparison to a number of these cytochromes in the main discussion.

Fig. 41: Redox Potential of Cytochrome c_{553} 

Cytochrome c_{553} in the presence of 0.1 mM Fe^{3+} /10 mM EDTA was titrated with μl additions of 100 mM ferrous ammonium sulphate. $\text{Log } (\text{ox})/(\text{red})$ cytochrome was plotted versus the observed potentials. The points are experimental, the line is of 60 mV slope and gives a midpoint potential for cytochrome c_{553} of +97 mV. The experimental points were determined from spectral changes (fig. 39) and the corresponding observed potentials.

CHAPTER V

STUDIES ON THE 165,000 g SEDIMENTABLE MATERIAL

A. The Separation of Two Chromatographic Cytochrome c Species

Redox potentiometric titrations (chapter III) indicated the presence of sedimentable low midpoint potential cytochromes c in the crude extract of Campylobacter sputorum subspecies mucosalis.

Examination of table 4 indicates the predominant presence of two species of cytochrome c (E_m -125 mV and -320 mV) in the sedimentable fraction. The following studies were undertaken with the aim of:

(a) the separation of these two species, and (b) the potentiometric characterization of the separated species.

The pellet obtained on centrifugation of the cell extract at 165,000 g was resuspended in 25 mM sodium phosphate buffer pH 7.0/ 100 mM NaCl. The suspended material was then centrifuged at 7,000 g for 20 minutes to remove turbidity. The clear solution so obtained was used for further studies, namely chromatography on sepharose 4B (CL) followed by chromatography on sephacryl S300 (fig. 42).

i. Chromatography on Sepharose 4B (CL)

Chromatography of the resuspended 165,000 g sedimentable material on sepharose 4B was carried out as described in fig. 43. The chromatographic pattern obtained shows a major protein fraction eluting first followed by a 410 nm absorbing peak. Of the latter fractions those giving a ratio of A_{410}/A_{280} of 0.5 or greater were pooled, concentrated on 6-aminohexyl sepharose and applied to a sephacryl S300 column.

ii. Chromatography on Sephacryl S300

Chromatography of concentrated sepharose fractions on sephacryl S300 was carried out as described in fig. 44. The chromatographic pattern is consistent with two overlapping peaks absorbing at 410 nm. Fractions giving a ratio of A_{410}/A_{280} of 0.7 or greater were pooled as shown in fig. 44 and concentrated on 6-aminohexyl sepharose.

Fig. 42.

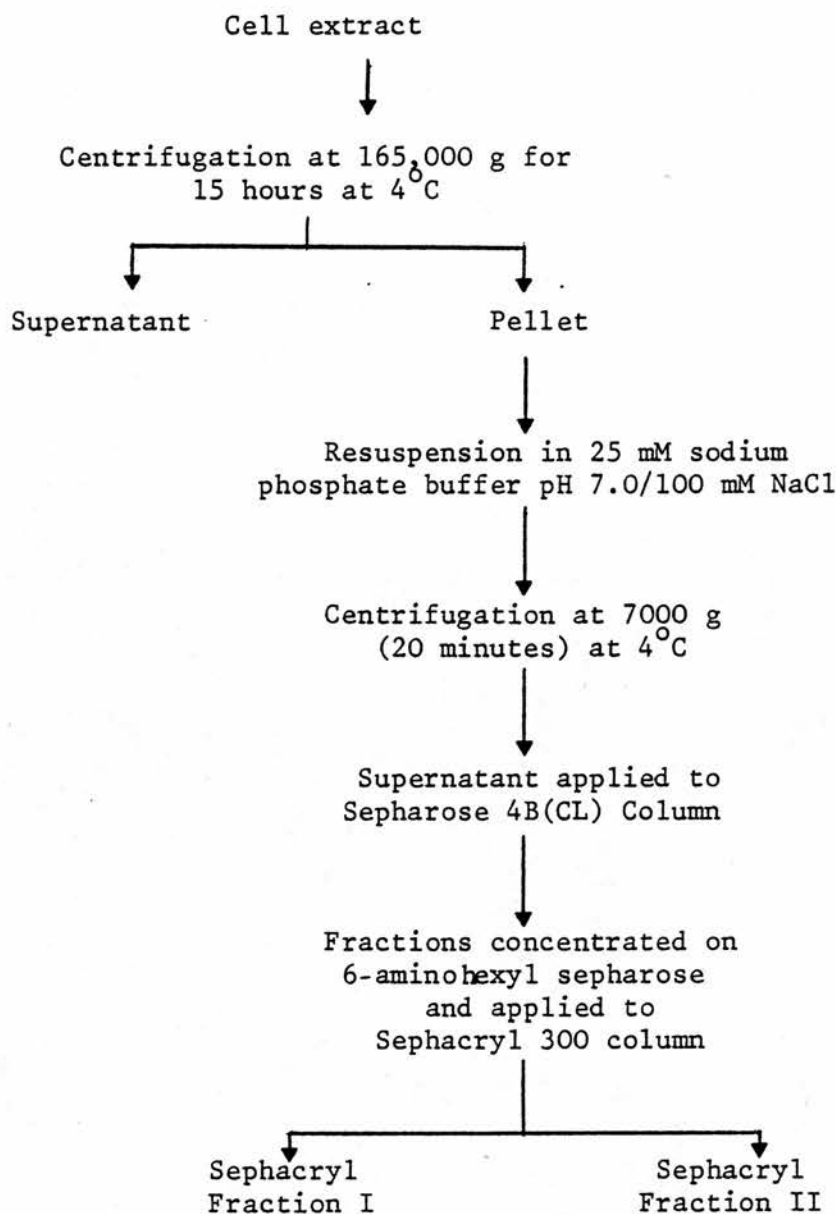


Fig. 43: Sepharose 4B(CL) Chromatography 165,000 g Sedimentable

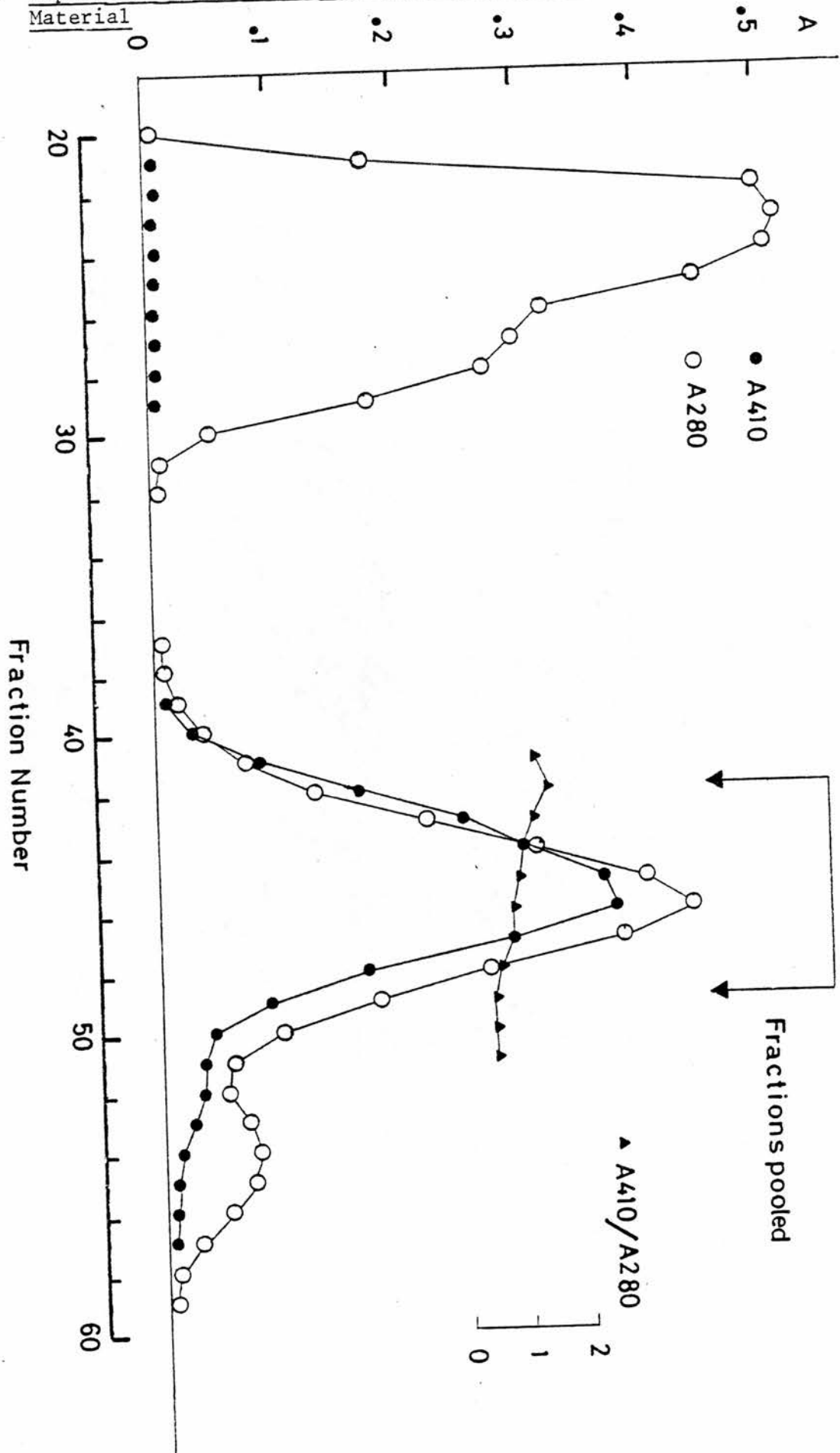


Fig. 43: Sepharose 4B(CL) Chromatography 165,000 g Sedimentable

Material

Chromatography was performed in a Sepharose 4B(CL) column 100 x 2.5 cm dimensions. Tris HCl pH 8.0/100 mM NaCl was used for equilibration and elution. The flow rate was 0.25 ml per minute. Arrows indicate fractions (each of 7.0 ml) pooled for further treatment.

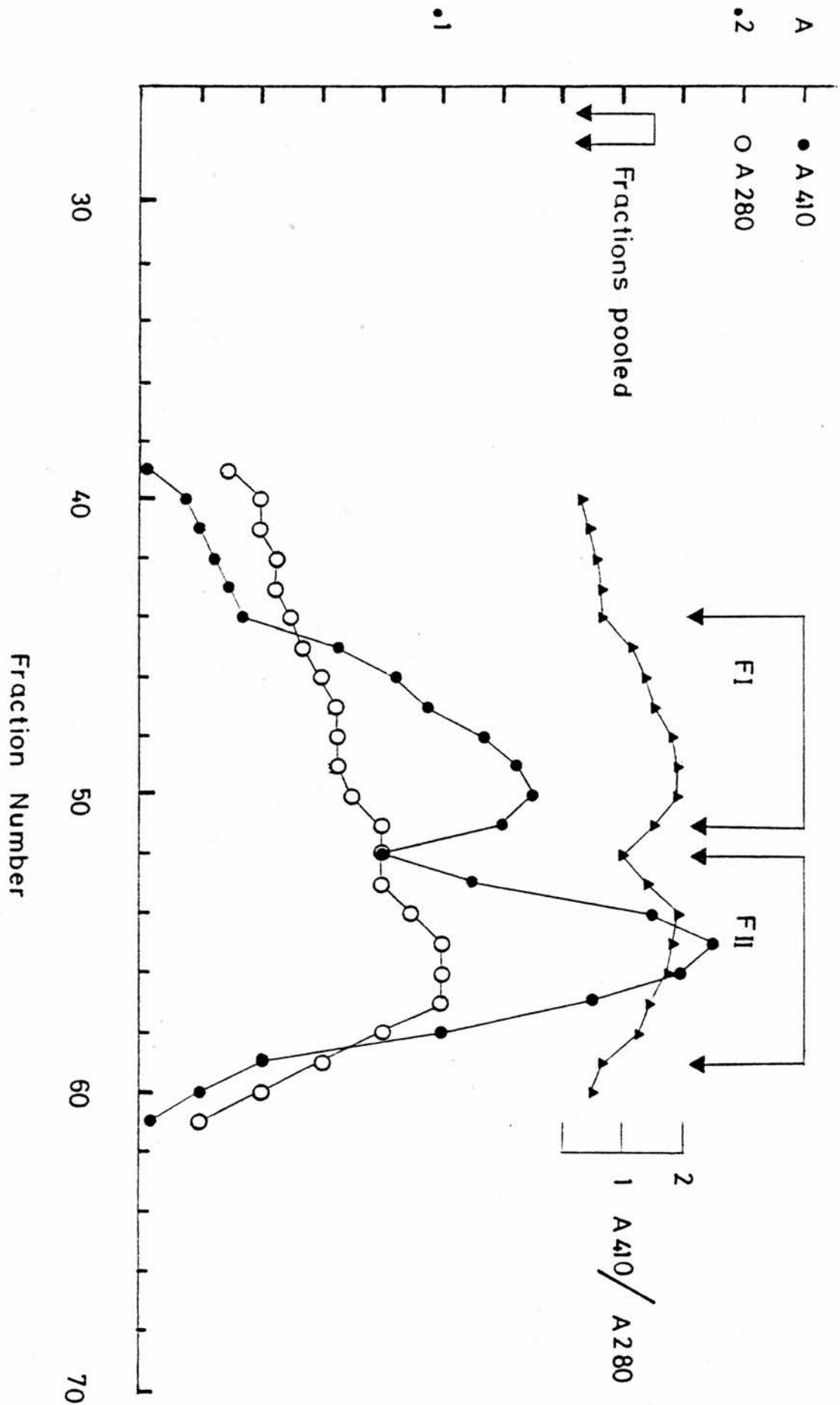
Fig. 44: Sephacryl S300 Chromatography of Concentrated SepharoseFractions

Fig. 44: Sephacryl S300 Chromatography of Concentrated Sepharose Fractions

Chromatography on Sephacryl S300 was performed in a column of 90 x 3.5 cm dimensions. Tris HCl pH 8.0/100 mM NaCl was used for equilibration and elution. The flow rate was 0.25 ml per minute. Fractions of 7.0 ml volume were pooled as indicated by the arrows. FI and FII refer to Sephacryl Fractions I and II respectively.

Table 10 lists the recoveries of the fractions as compared to the 165,000 g sedimentable material.

iii. Concentration of Chromatographic Fractions

Pooled chromatographic fractions were adjusted to pH 7.0 with 1M acetic acid and dialyzed against 3 changes of 10 times their volume of 10 mM sodium phosphate buffer pH 7.0. The dialyzed material was then applied to a small 6-aminohexyl sepharose column of 3 ml bed volume equilibrated with 10 mM sodium phosphate buffer pH 7.0. The adsorbed material was eluted with 100 mM Na phosphate buffer pH 7.0.

B. Redox Potentiometric Studies

The partially separated sephacryl fractions termed Sephacryl Fraction I and II were examined by redox potentiometry. A working hypothesis was that they may represent the two main potentiometric species observed in redox titrations of the starting material.

However, due to incomplete chromatographic resolution "potentiometric" homogeneity was not expected.

i. Redox Potentiometry of Sephacryl Fraction I

Redox potentiometry of Sephacryl Fraction I was carried out as described in fig. 45. Plotting the potentials observed vs log the ratio of oxidized cytochrome to reduced cytochrome gave a sigmoid curve indicative of the presence of two potentiometric species (fig. 46). The experimental curve closely fits a theoretical curve of two components of midpoint potentials of -170 mV and -290 mV (fig. 47) contributing to the total absorbance at 553 nm in the ratio of (1.24:1) equivalent to 55% and 45% of total cytochrome respectively.

ii. Redox Potentiometry of Sephacryl Fraction II

Redox potentiometry of Sephacryl Fraction II was performed as described in fig. 48. The titration curve (fig. 49) did not exhibit a 60 mV slope but was consistent with the presence of 3 potentiometric

Table 10: Recoveries of Sephacryl Fractions I and II

<u>Fraction</u>	<u>nmol</u>	<u>%</u>
Resuspended 165,000 g sedimentable material	600	100
Sepharose 6B Fractions	450	75
Sephacryl Fraction I	110	18
Sephacryl Fraction II	180	30

species of midpoint potentials $x > -150 \text{ mV} > y$ contributing to the total absorbance in the ratios 1:4.33:1.2 equivalent to 15.3%, 66.3% and 18.7% of total cytochrome in fraction II respectively. x and y are unknown and only the predominant species of midpoint potential of -150 mV could be resolved (fig. 49) and the corresponding 60 mV line drawn. Not enough titration points were obtained to enable the characterisation of the other two components.

C. SDS Gel Electrophoresis of Sephadryl Fraction I and Fraction II

SDS electrophoresis of Sephadryl Fractions I and II was performed as described in fig. 50. Two major and one minor electrophoretic bands absorbing at 410 nm were obtained on SDS gels for Sephadryl Fraction I (fig. 50); while Sephadryl Fraction II gave only one such band (fig. 51). The minor band of Fraction I and the band of Fraction II correspond to a molecular weight of 50,000. Molecular weights of 110,000 and 200,000 were obtained for the major bands of Fraction I by extrapolation of the calibration graph (fig. 52) to regions of higher molecular weight. Since molecular weight markers of such molecular weights were not included in the gels the figures of 110,000 and 200,000 obtained can at best be regarded as only approximate.

The SDS gels moreover indicate that considerable purity was achieved (fig. 50; fig. 51).

D. The Spectra of Sephadryl Fractions I and II

The absorption spectra of Sephadryl Fractions I and II are shown on fig. 53 and fig. 54. The spectra are very similar except that the spectrum of Fraction II does not exhibit a maximum in the ultraviolet region around 280 nm, the absorbance decreases rather smoothly between 250 nm and 310 nm. Absorption maxima at 695 nm and 630 nm are absent. The four pyrrole nitrogens provide planar ligands for the haem iron and

Fig. 45: Redox Titration Spectra Sephadryl Fraction I

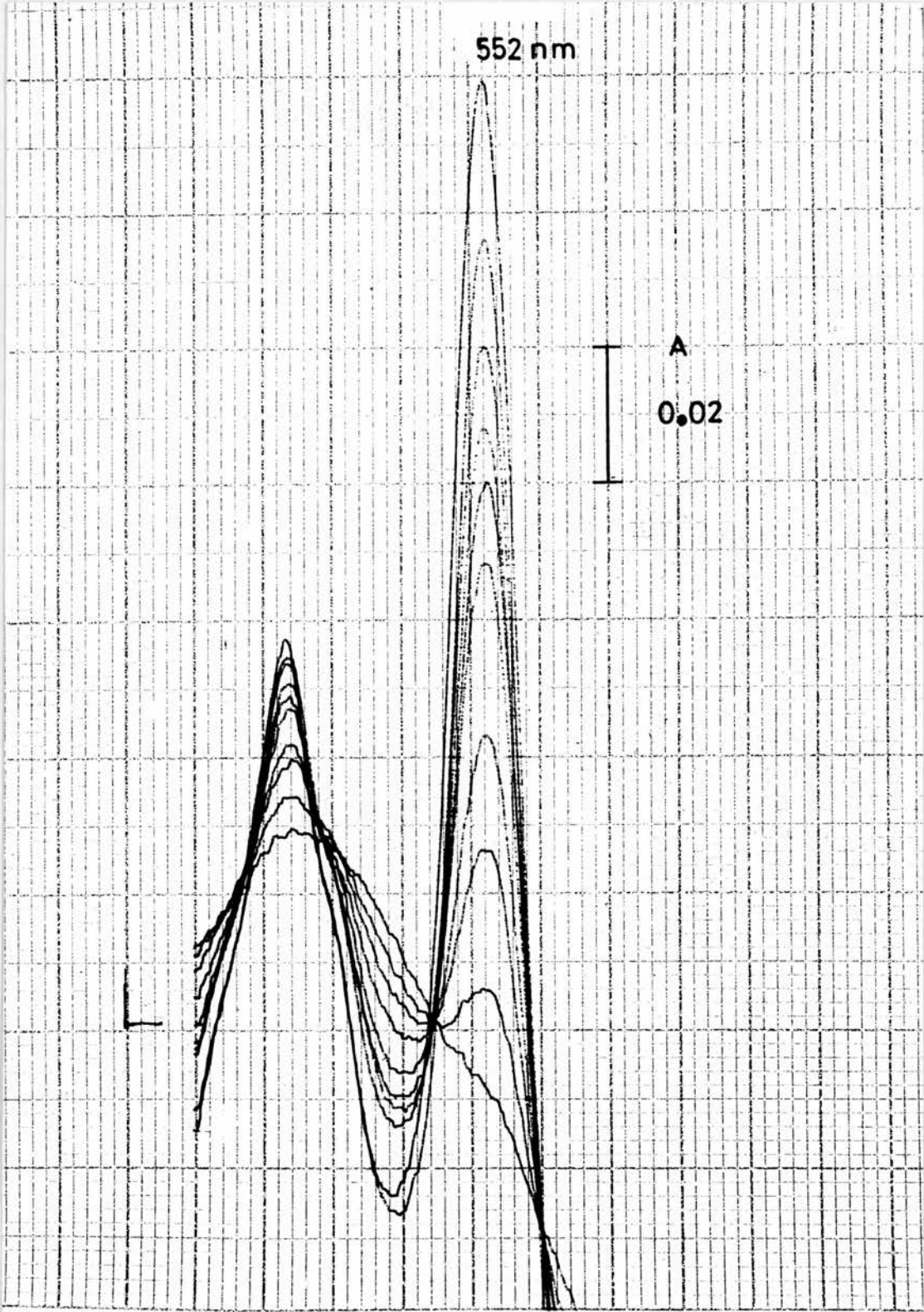
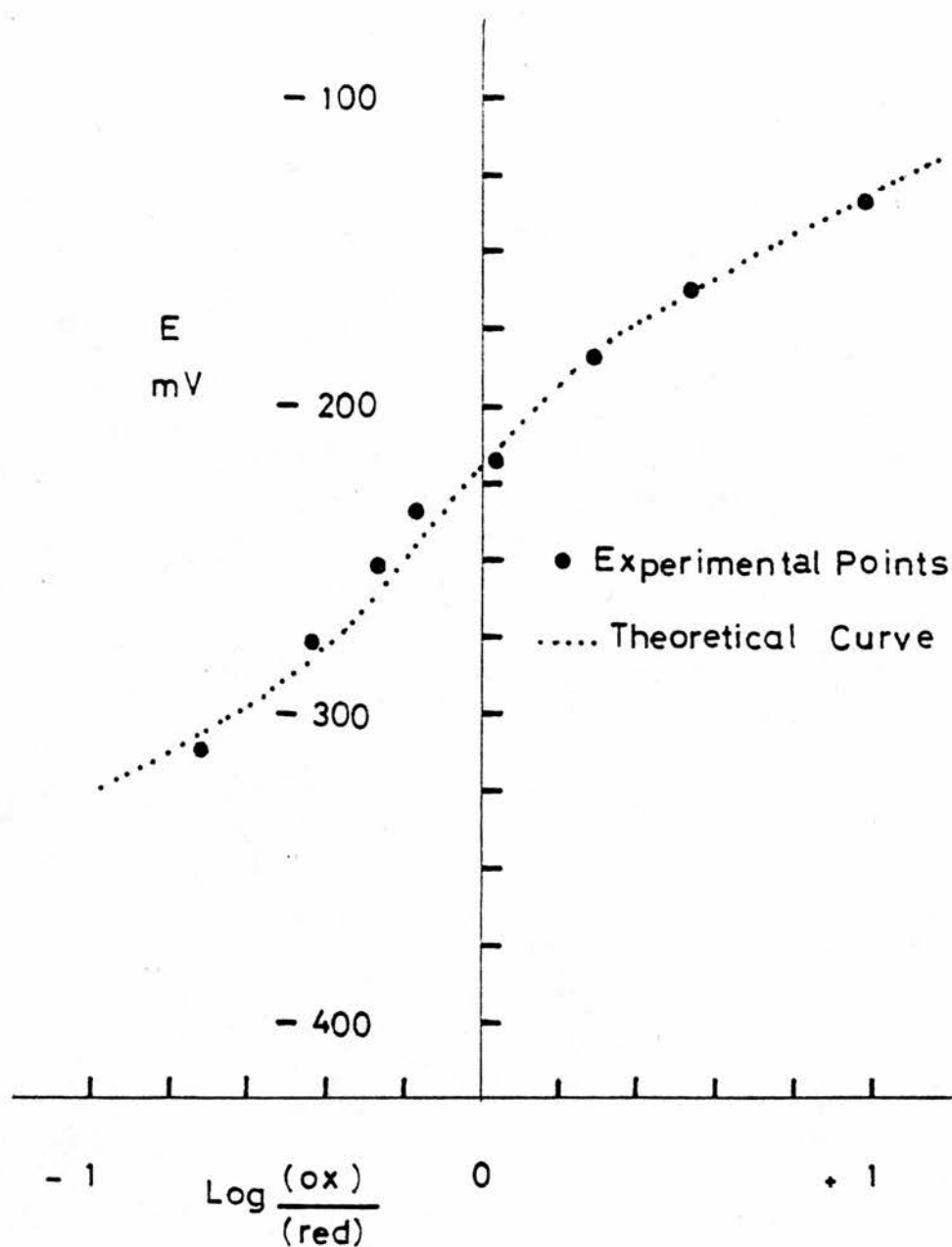


Fig. 45: Redox Titration Spectra Sephacryl Fraction I

Redox potentiometry of Sephacryl Fraction I was performed as described under materials and methods. Anaerobic conditions were achieved by equilibration with argon for one hour. Sodium dithionite (100 mM) was used as a reductant. PES, PMS, BV, HNQ, FMN, DQ and DAD were used as redox mediators at a final concentration of 10 μ M; Fe^{3+} EDTA at a final concentration of 20 μ M.

Sodium dithionite (E_m -660 mV) was chosen as titrant since the presence of a component with midpoint potential more negative than -300 mV which is the lower limit for NADH titrations, was expected in Sephacryl Fraction I. Spectra were recorded as described in methods.

Fig. 46: Titration Curve Sephacryl Fraction I

Experimental points were obtained from the spectra (fig. 45) and the observed potentials. The theoretical curve was calculated from the resolved components of fig. 47.

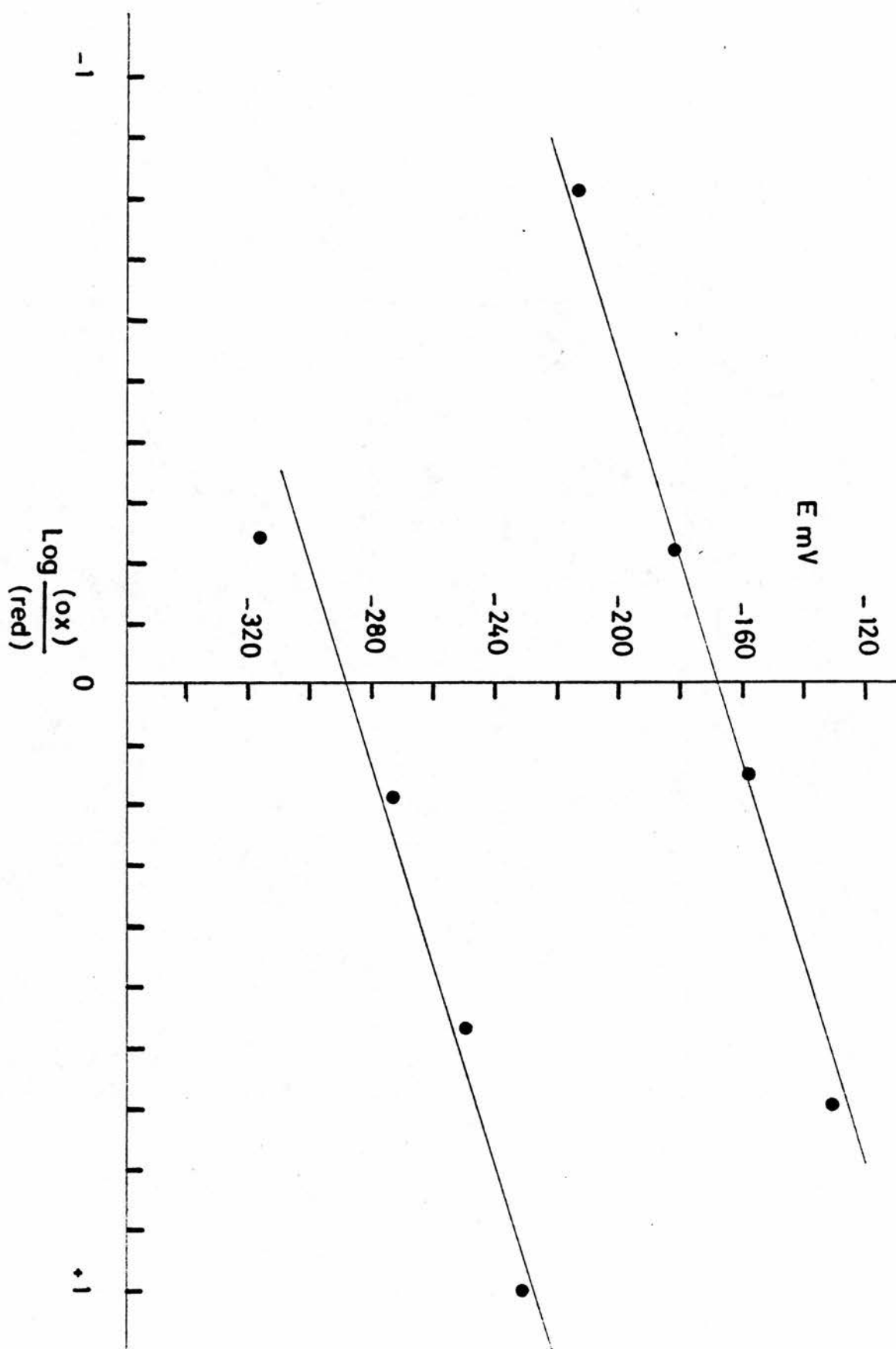
Fig. 47: Resolved Components - Redox Titration Sephacryl Fraction I

Fig. 47: Resolved Components - Redox Titration Sephacryl Fraction I

The redox titration curve of Sephacryl Fraction I was resolved by means described earlier (Methods) into two components.

The theoretical curve fits the experimental curve closely (fig. 46).

The resolved components exhibits E_m values of -170 mV and -290 mV

(fig. 47). The lines are 60 mV lines.

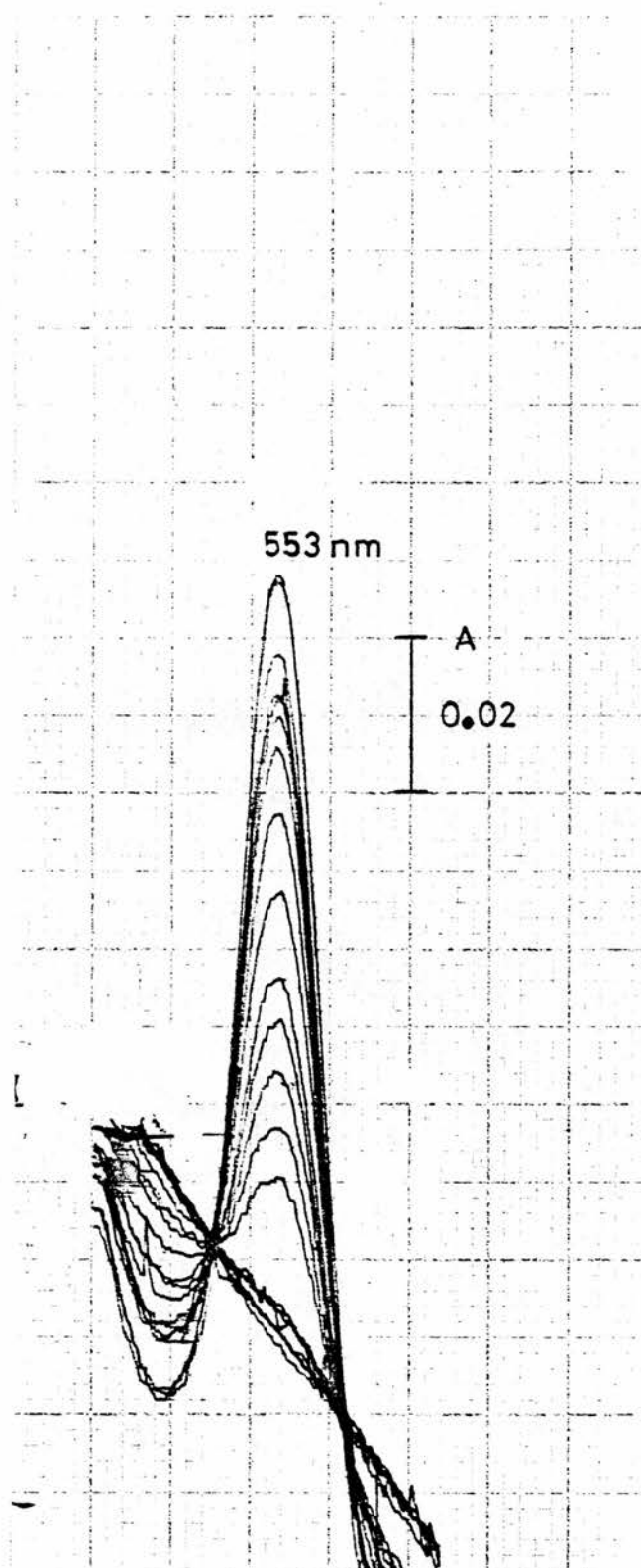
Fig. 48: Redox Titration Spectra Sephacryl Fraction II

Fig. 48: Redox Titration Spectra Sephadex Fraction II

Redox potentiometry of Sephadex Fraction II was carried out under anaerobic conditions as described under Methods. The cuvette contained 0.5 mg NADH cytochrome c reductase. NADH (150 mM) was used as titrant. PES, PMS, HNQ, BV, FMN, DQ and DAD were used as redox mediators at a final concentration of 10 mM; Fe^{3+} EDTA at 20 μM . Equilibration with argon gas was allowed to proceed for one hour before titration was performed. NADH ($E_m -320$) was found by trial to be suitable for titration of Sephadex Fraction II.

Spectra were recorded as described in Methods.

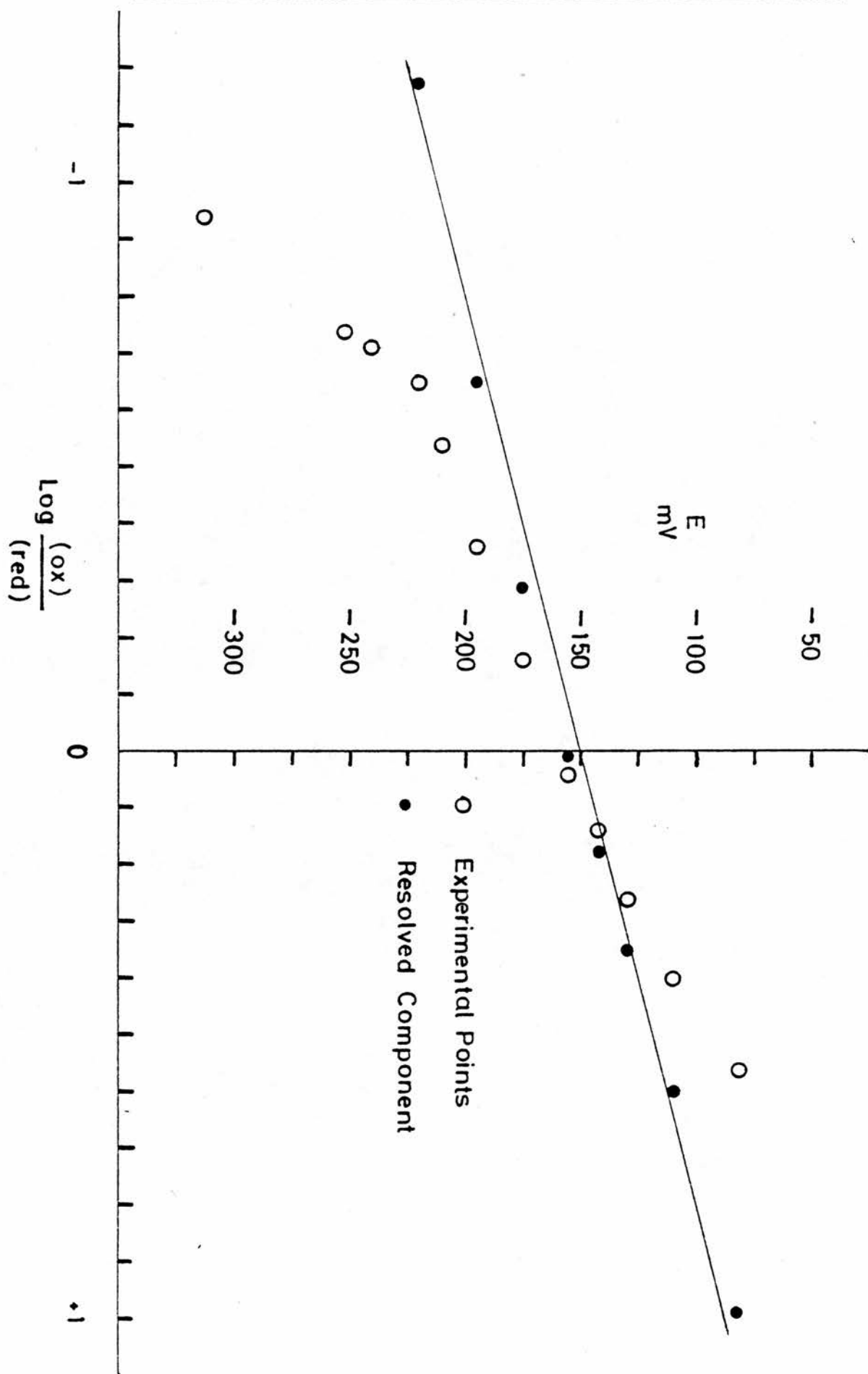
Fig. 49: Resolved Components - Redox Titration Sephacryl Fraction II

Fig. 49: Resolved Components - Redox Titration Sephadryl Fraction II

The titration curve Sephadryl Fraction II (O) could be resolved into a dominant component (●) of E_m -150 mV (text). A component of higher E_m was assumed to be totally reduced and a component of lower E_m to be totally oxidized during the titration of the dominant component.

The experimental points were obtained from the spectra of fig. 48 and the observed potentials. The line is a 60 mV line.

Fig. 50: SDS Electrophoresis Sephacryl Fraction I (SFI)

SDS electrophoresis of Sephacryl Fraction I (SFI) containing 3 nmol cytochrome was carried out in 7.5% gels. The appropriate amount of acrylamide/bisacrylamide was polymerized and electrophoresis performed as described in materials and methods. 1 nmol horse heart cytochrome c (HHCC) was included as an internal marker. Three bands absorbing at 410 nm could be identified corresponding to the molecular weight of approximately 200,000 (A), approximately 109,000 (B) and 50,000 (C) (fig. 52).

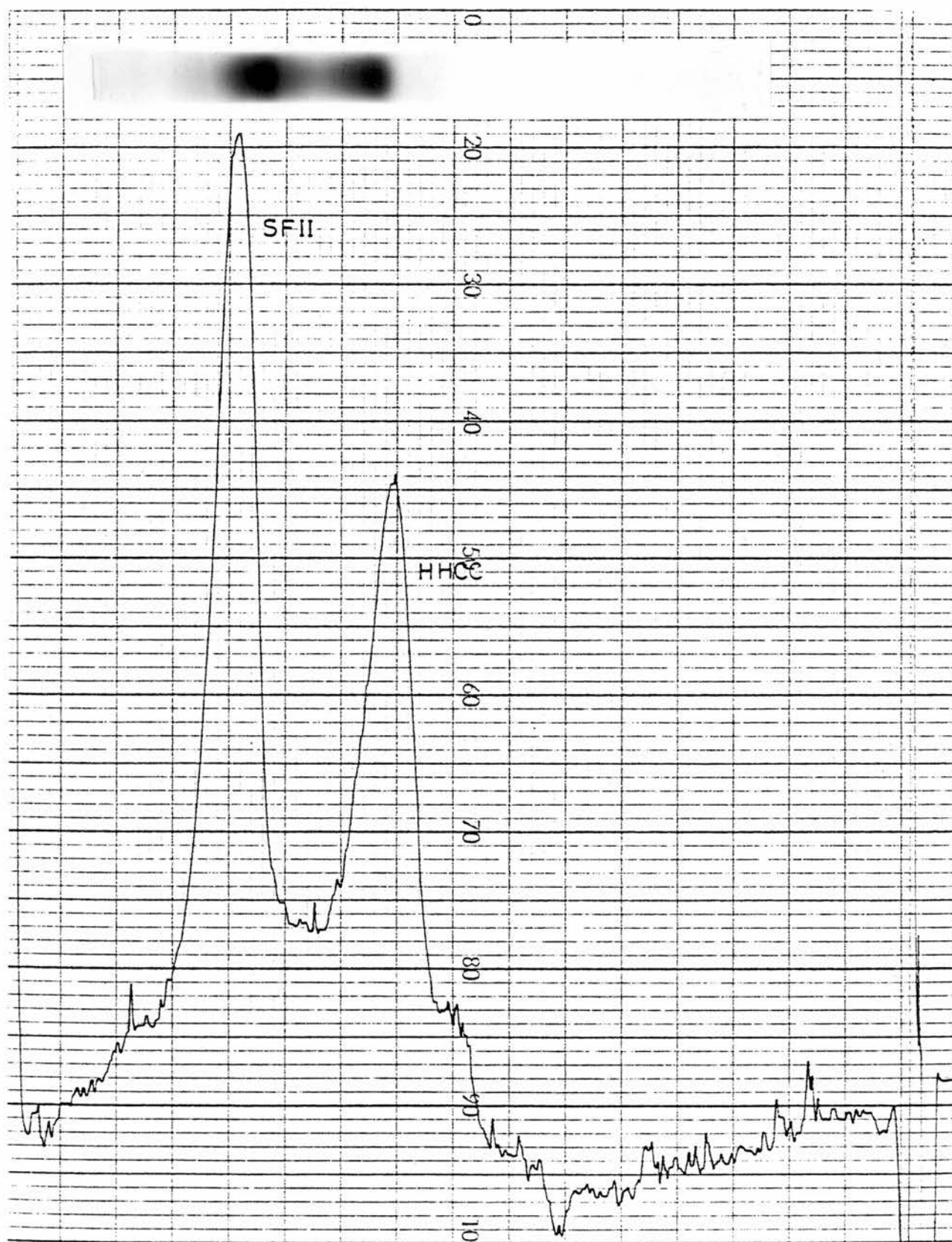
Fig. 51: SDS Electrophoresis: Gel Scan (410 nm) - Sephacryl Fraction II

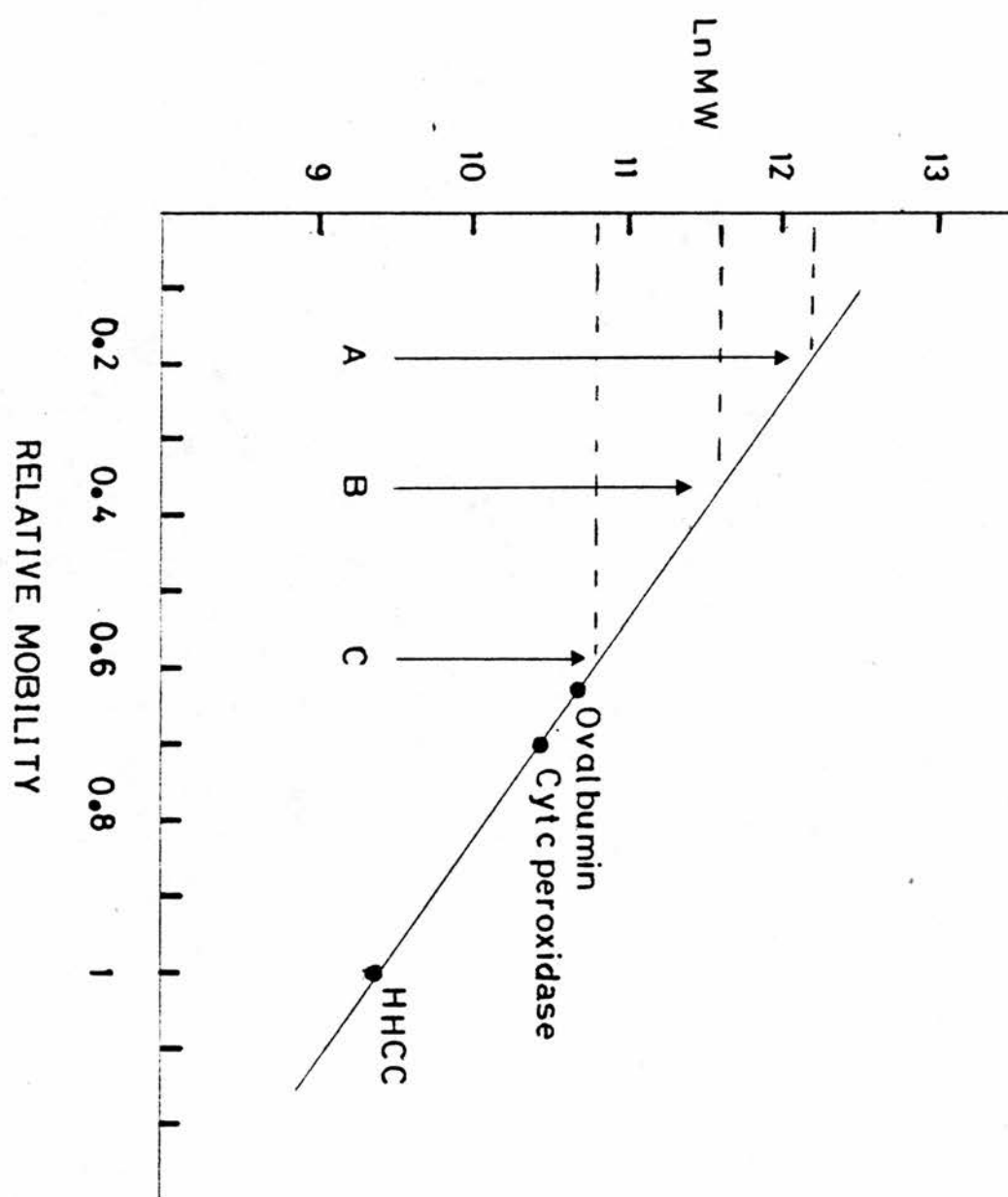
Fig. 52: The Molecular Weights of Sephacryl Fractions I and II

Fig. 52: The Molecular Weights of Sephacryl Fractions I and II

The molecular weights of the cytochrome species in Sephacryl Fractions I and II were estimated from mobility on SDS gels relative to horse heart cytochrome c (HHCC). Ovalbumin and cytochrome c peroxidase were used as molecular weight markers. Three bands A, B and C were identified on Sephacryl Fraction I. Only one band corresponding to Sephacryl Fraction I - band C was identified on SDS electrophoresis of Sephacryl Fraction II. The corresponding molecular weights are approximately 200,000, approximately 109,000 and 50,000.

the fifth ligand is usually a histidine in c-type cytochromes. The sixth ligand in the low spin cytochromes c is generally methionine although cytochromes c_3 have histidine. The high spin proteins such as the cytochromes c' and the globins have no sixth iron ligand. Absence of absorption maxima at 695 nm is indicative of the absence of methionine coordination to the iron. The absence of absorption maxima at 630 nm is, on the other hand, indicative of a low spin character. Hence the cytochromes of Sephadex Fractions I and II are low spin cytochromes and by analogy with the cytochrome c_3 and cytochrome b_5 may have their haem iron present as a bis histidinyll adduct (Figs. 55 and 56).

The absorption maxima and absorption ratios of Sephadex Fractions I and II are shown in table 11. Due to the partial purity of the preparations these figures must be regarded as tentative.

E. Discussion

The chromatographic and potentiometric data presented in this section clearly indicate that partial separation of potentiometrically distinct species of cytochrome c in the 165,000 g sedimentable fraction of the cell extract of Campylobacter sputorum subspecies mucosalis was possible. It is proposed that complete separation of at least the species exhibiting a midpoint potential of -150 mV is attainable, resulting in a potentiometrically homogeneous species. It has not been possible to separate the components of Sephadex Fraction I exhibiting midpoint potentials of -170 mV and -290 mV.

The different molecular weights obtained on SDS are consistent with the presence of different potentiometric species i.e. the -150 mV species appears to have a molecular weight of 50,000 while the -170 and -290 mV components appear to be of higher molecular weight and therefore distinct.

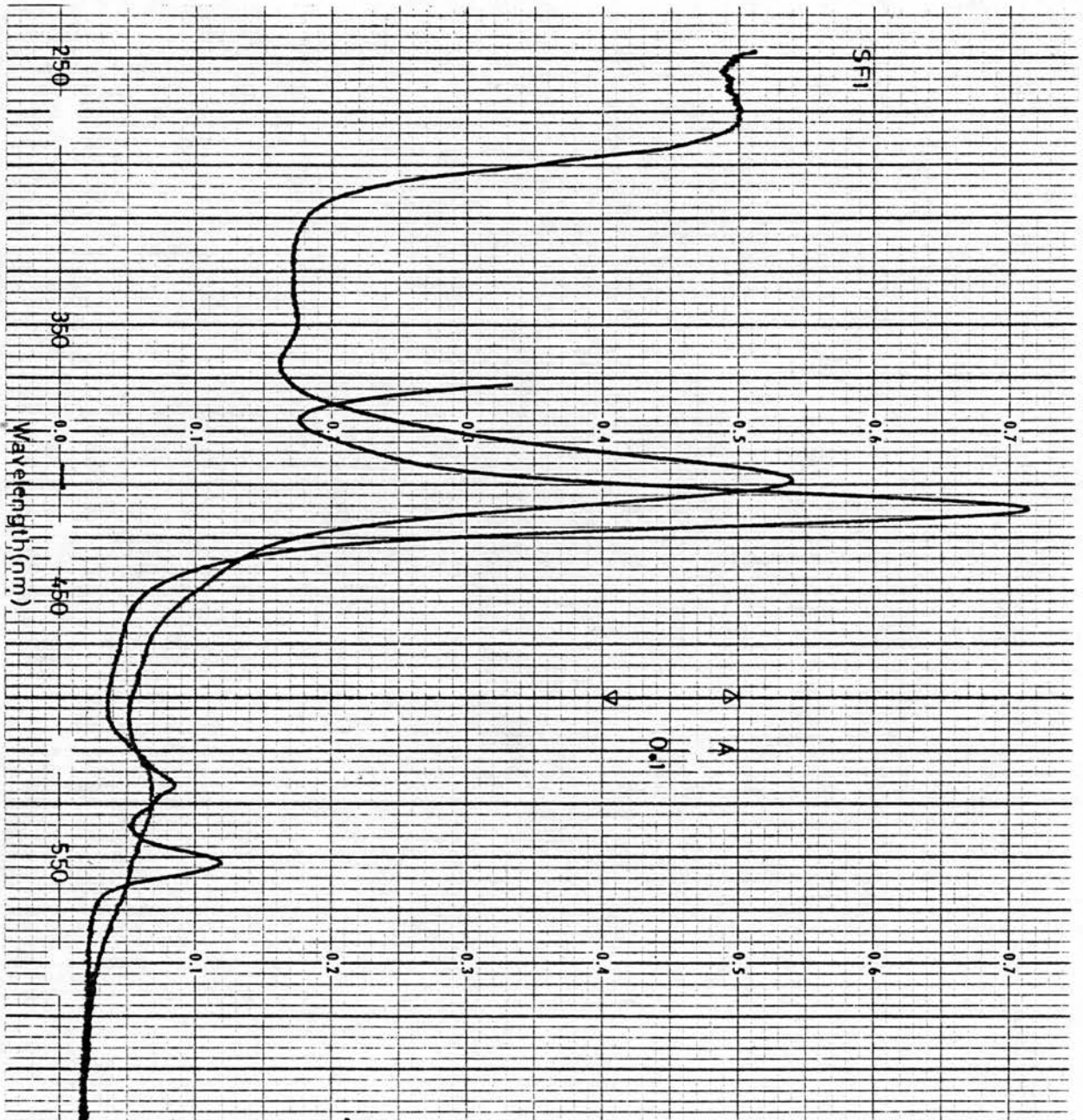
Fig. 53: Absorption Spectra Sephacryl Fraction I

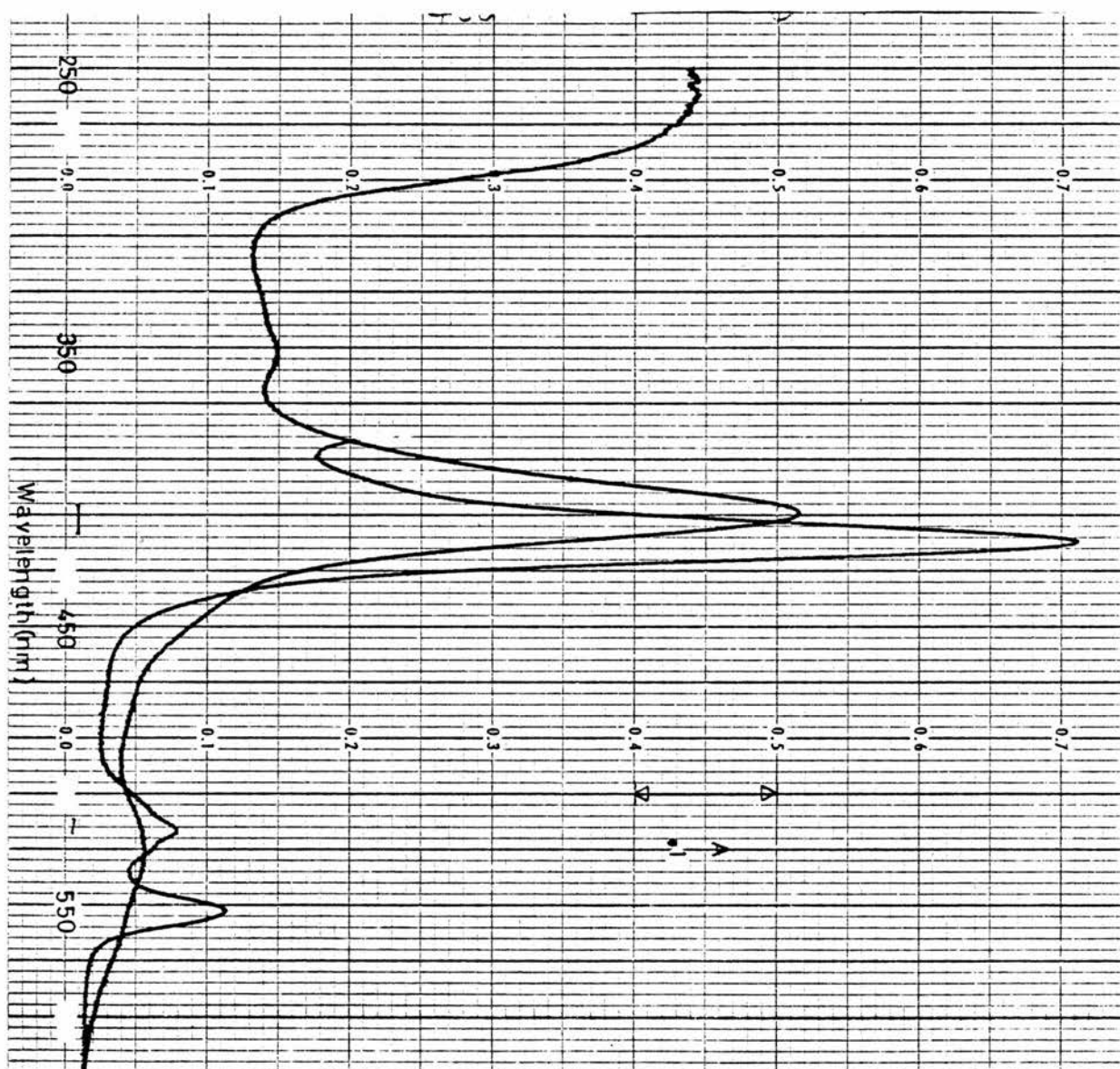
Fig. 54: Absorption Spectra Sephacryl Fraction II

Fig. 55: Absorption Spectra Sephadryl Fraction I

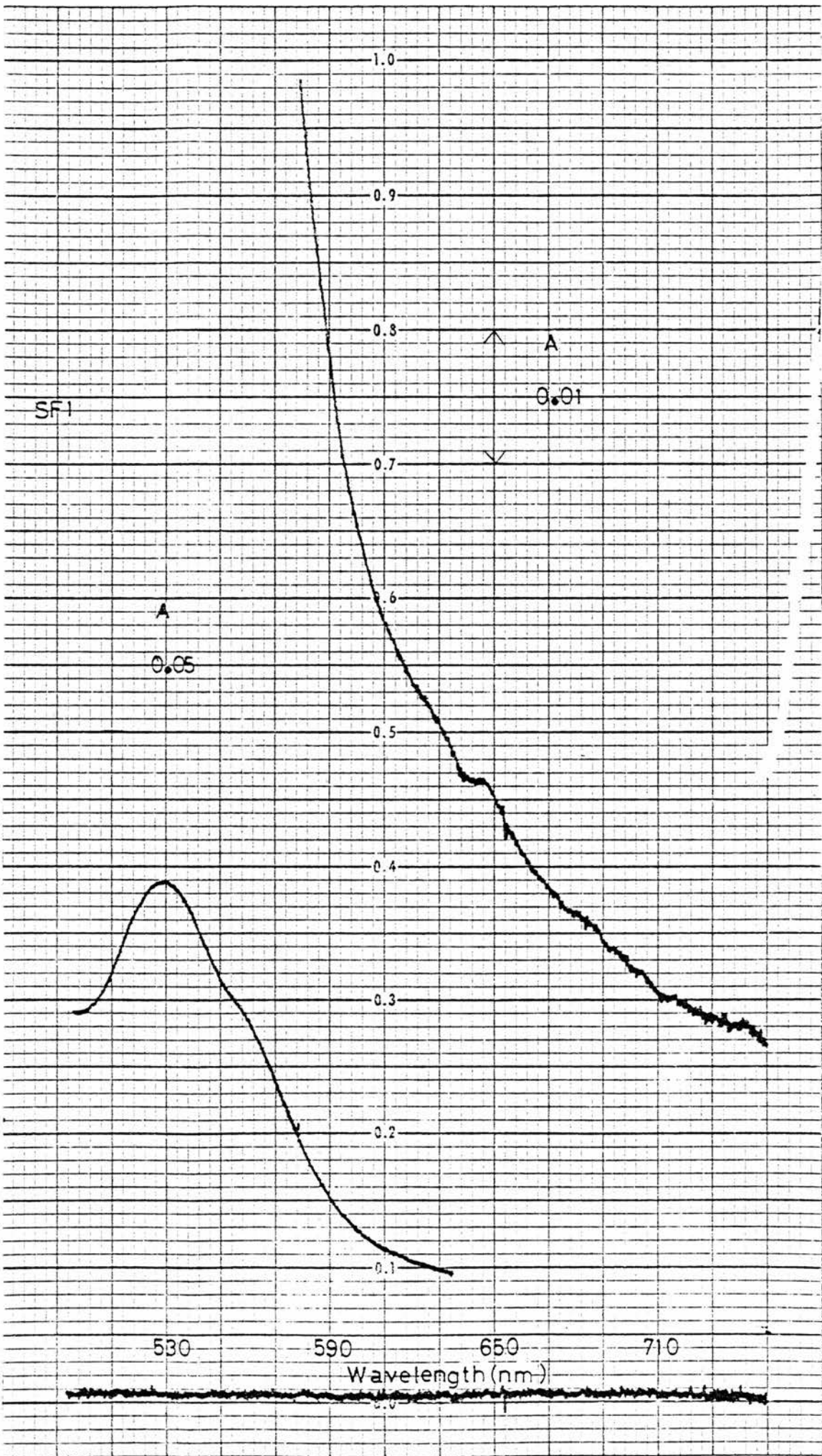
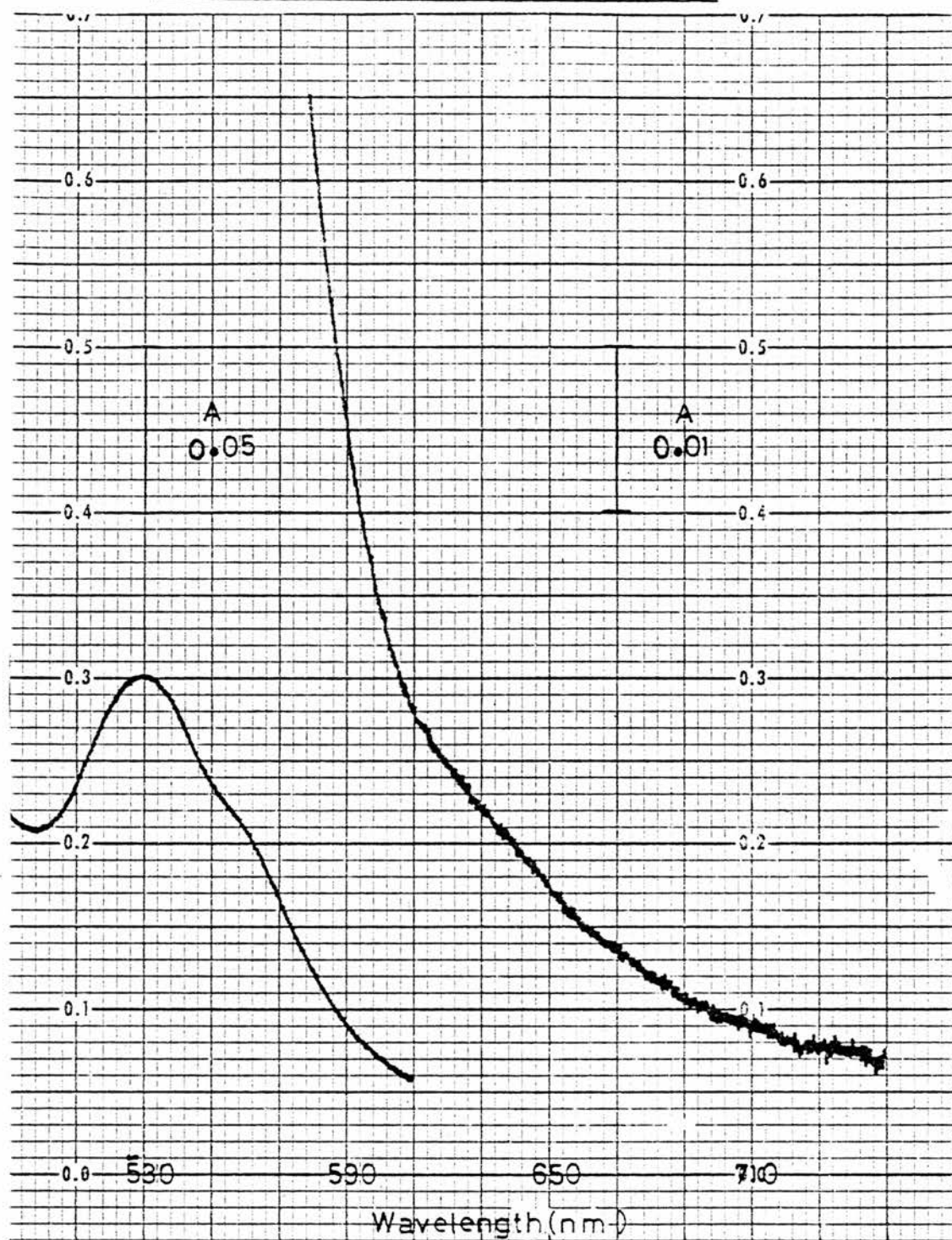


Fig. 56: Absorption Spectra Sephacryl Fraction II



The potentiometric results obtained in this chapter are presented in table 12 together with those obtained for the low midpoint potential cytochromes from redox titrations of the 100,000 g sedimentable material. The potentiometric identification of the component with an E_m -170 mV in Sephacryl Fraction I but not in the 100,000 g sedimentable fraction can be rationalized in the following manner:

- a) in the titrations of the crude preparations 2 components with E_m values differing by 25 mV would titrate almost as one component; and the mathematical resolution of components with E_m values differing by less than 40 mV is difficult even when using computer analysis. Thus it is proposed that the component (E_m -170) mV titrated as part of the component (E_m -150) mV identified in Sephacryl Fraction II.
- b) Interference by minor component(s) of less negative midpoint potential(s), experimental error and the limitations of the mathematical methods used to resolve the complex curves probably led to less negative values being deduced for the combined component (E_m -170/ E_m -150) in the crude fractions.

Despite partial purity the absorption spectra of Sephacryl Fractions I and II serve to differentiate the cytochrome present from other low spin cytochromes c of similar low midpoint potentials. This will be treated in greater detail within the main discussion.

Table 11: Absorption Maxima and Absorption Ratios Sephacryl
Fractions I and II

	<u>Fraction I</u>	<u>Fraction II</u>
γ_{ox}	406 nm	406 nm
γ_{red}	427 nm	427 nm
α	551.5 nm	551.5 nm
β	522.5 nm	522.5 nm
γ_{red}/γ_{ox}	1.36	1.38
γ_{red}/α	5.53	6.16
γ_{ox}/A_{274}	0.975	1.23
α/β	1.36	1.45
α/A_{274}	0.24	0.28

Table 12: Proposed Relationships of Identified Potentiometric
Species

	<u>mV</u>	<u>mV</u>
Cell extract	-125	-310
	↓	↓
100,000 g sedimentable	-125	-320
	↓	↓
Sephacryl Fraction I	-170	-290
	↓	
Sephacryl Fraction II	-150	

CHAPTER VI

DISCUSSION

Discussion

The term cytochrome c originated on the basis of both structural and spectral properties i.e. the mode of attachment of a haem prosthetic group to an apoprotein and the absorption characteristics of the haem protein so formed. The cytochromes c are redox proteins and the present study was concerned with the potentiometric identification of the c-type cytochromes of Campylobacter sputorum subspecies mucosalis, followed by their isolation and characterization. A brief discussion of the potentiometric characterization of cytochromes will be followed by a section dealing with the properties of cytochrome c₅₅₃. The low midpoint potential cytochromes c of Campylobacter sputorum subspecies mucosalis will be discussed and in a final section the electron transport system of Campylobacter sputorum subspecies mucosalis will be compared to those of other organisms.

A. The Potentiometric Characterization of Bacterial Cytochromes

A review of the literature shows that redox potentiometry has been utilized in two different manners to characterize bacterial cytochromes. It has either been used to determine the number and midpoint potentials of cytochromes which constitute a bacterial respiratory system; or to determine the midpoint potentials of purified cytochrome components.

The first approach entails the use of crude cell extracts of bacterial cells. This approach has not usually been followed by the isolation of the potentiometrically identified components. Such studies were, however, important in establishing that different redox potentiometric species of cytochromes occur in bacteria under different growth conditions, particularly when the terminal electron acceptor is varied. For example Hendler et al. 1975 and Pudek and Bragg 1976

have utilized this approach in attempting to characterize the cytochromes of E. Coli grown under aerobic conditions; while Reid and Ingledew 1978 extended the method to cells grown anaerobically with either fumarate or nitrate as terminal acceptors, and Hackett and Bragg 1982 to cells grown at 28°C and 42°C.

Many workers on the other hand have measured the midpoint potentials of purified cytochromes (e.g. Meyer et al. 1968; Shioi et al. 1972; Yagi 1979; Fiechtner and Kassner 1979), without establishing whether the potentiometric species they characterized can be identified in the intact system.

The present study was a combination of both of these approaches i.e. an attempt was made to potentiometrically identify the cytochromes c in the crude preparations of Campylobacter sputorum subspecies mucosalis, and to isolate these in potentiometrically homogeneous forms. The importance of combining both these approaches has been fully justified by the data obtained. Titration of crude extracts indicated the presence of at least 4 c-type cytochromes when earlier spectral studies had only indicated one (Lawson et al. 1981). However by separation of the cytochromes c and titration of the separated components it was possible to show the presence of at least 5 c-type cytochromes.

In general redox potentiometry has the advantage of being more resolving than simple spectral characterization. As a further example, spectral studies suggested that cytochrome $b_{556}^{NO_3}$ is the direct electron donor to nitrate reductase of E. Coli. However, Hackett and Bragg (1982) have identified two cytochromes b with midpoint potentials of +10 mV and +125 mV in the purified nitrate reductase of E. Coli. Nevertheless redox potentiometry provides only preliminary information with respect to the organization and sequence of bacterial electron transport chains. Kinetic studies and the utilization of electron

transport inhibitors are necessary in addition to redox potentiometry if complete characterization of an electron transport chain is to be achieved.

B. The Properties of Cytochrome c_{553}

i. Physicochemical Properties

Campylobacter cytochrome c_{553} shows considerable similarities to Chloropseudomonas ethylica cytochrome c_{555} , Chlorobium thiosulphatophilum cytochrome c_{555} and Desulfovibrio desulfuricans cytochrome c_{553} .

The spectrum of Campylobacter cytochrome c_{553} was shown in fig. 28.

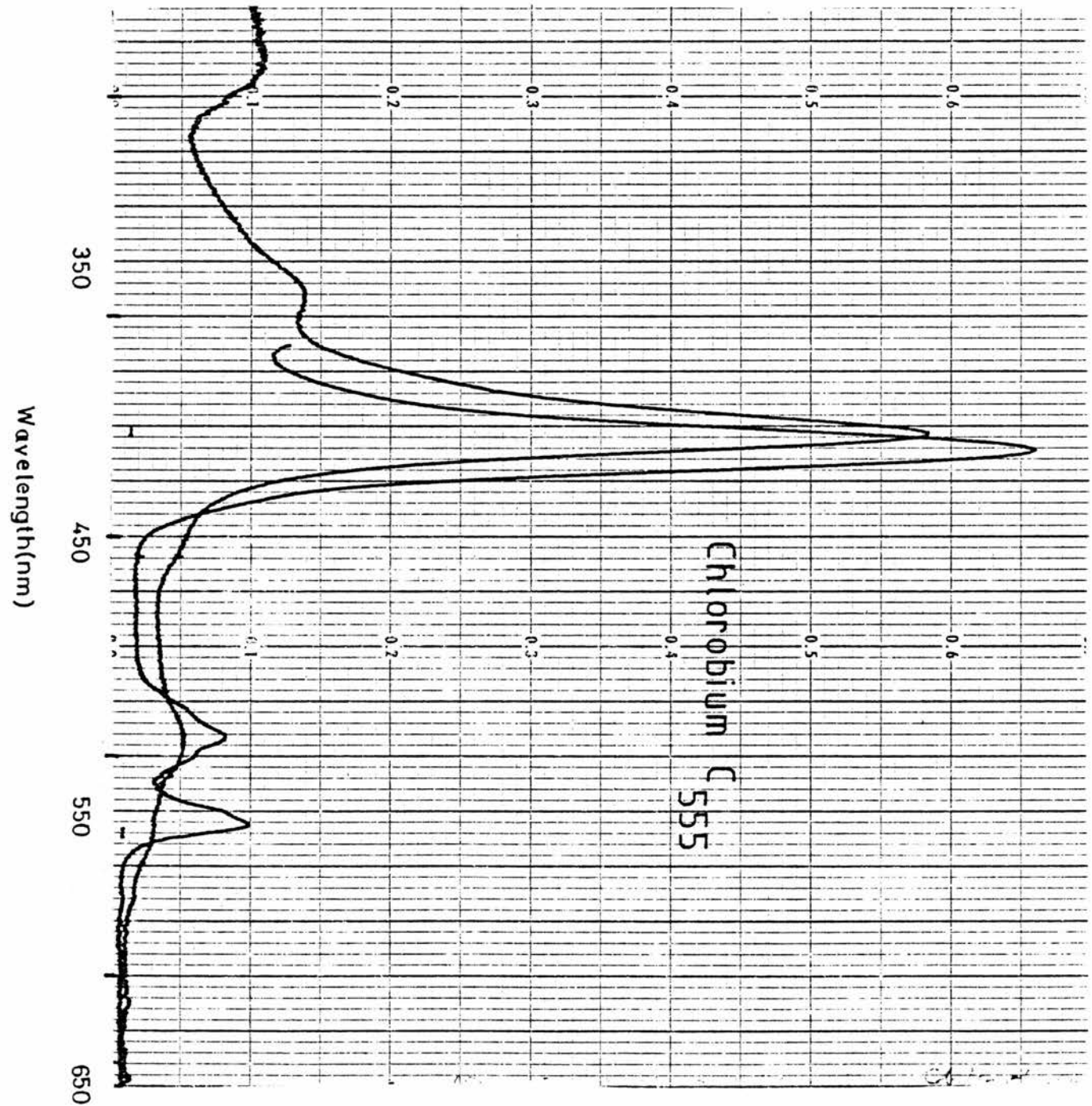
Fig. 57 shows the spectrum of Chlorobium thiosulphatophilum c_{555} and table 13 lists properties of these cytochromes. The absorption spectra of these cytochromes are almost identical. The reduced α -peak at 553-555 nm is asymmetric with a shoulder at 548-550 nm; the β -peak is at 522-523 nm and the reduced soret is at 416-418 nm. The absorption ratios are also similar. The $\gamma_{\text{red}}/\alpha$ is greater than 7 as compared to 5 for mitochondrial cytochrome c and Pseudomonas aeruginosa c_{551} .

The α/β ratio (1.2) is less than those reported for Pseudomonas c_{551} and mitochondrial cytochrome c (1.7 and 1.9 - respectively).

The mid potentials of the cytochromes fall within the range of +50 to +114 mV. These midpoint potentials are however rather low in comparison with those reported for Pseudomonas c_{551} and mitochondrial cytochrome c. With the exception of Chlorobium cytochrome c_{555} the isoelectric points of the cytochromes fall on the acidic side of neutrality. This is rather characteristic of most bacterial cytochromes c in contrast to the mitochondrial cytochromes c which are basic. The molecular weight of the cytochromes are similar to that of Pseudomonas cytochrome c_{551} , the exception being Chloropseudomonas cytochrome c_{555} which has a molecular weight very similar to that of mitochondrial cytochrome c.

Table 13: The Properties of Some Low Spin High Potential Cytochromes c

Cytochrome	Absorption Maxima				Absorbance Ratio			M.W.	References
	α	β	γ_{red}	γ_{red}/α	α/β	E_m mV	PI		
<u>Campylobacter c₅₅₃</u>	553 (548)	522	416	7.69	1.27	+103	acidic	8714	This thesis
<u>Chloropseudomonas c₅₅₅</u>	555 (550)	523	417.5	7.52	1.19	+103	4.65	12000	Shioi et al. 1972
<u>Chlorobium c₅₅₅</u>	555 (550)	528	418.5	7.02	1.20	+145 (pH 6) +114 (pH 8)	10.5	9970	Meyer et al. 1968 Yammanaka and Okunuki 1968 Meyer 1970
<u>D. desulfuricans c₅₅₃</u>	553 (550)	525	417	7.66	1.22	50-20 ⁺	6.6	9381	Fauque et al. 1979
<u>Pseudomonas c₅₅₁</u>	551	521	416	4.9- 5.1	1.6- 1.7	+286 (pH 6.5)	4.7	8000	Horio et al. 1960 Horio 1958a and b Ambler 1963
Mitochondrial cytochrome c	550	521	415	5	1.9	+260	10.5	12200	Lemberg and Barrett 1973 Dickerson and Timkovich 1975

Fig. 57: Absorption Spectra Chlorobium ThiosulphatophilumCytochrome c₅₅₅

ii. The Amino Acid Composition of Cytochrome c₅₅₃

Three amino acids, As(x), alanine and lysine dominate the amino acid composition of cytochrome c₅₅₃. Two amino acids are notably absent: arginine and tryptophan. Arginyl residues are reported not to be conserved in bacterial cytochromes unlike eukaryotic cytochromes (Dickerson and Timkovich 1975). By contrast an important structural and functional role has been suggested for tryptophan (Salemme 1977; Aviram and Schejter 1971). Amino acid sequences and tertiary structure studies on eukaryotic and prokaryotic cytochromes c have revealed invariant residues at structurally equivalent positions. One of these is the tryptophan corresponding to position 59 in mitochondrial cytochrome c and analogous positions in other cytochromes of low spin and high midpoint potential (Dickerson and Timkovich 1975; Salemme 1977). The available x-ray analysis show that this tryptophan is hydrogen bonded to the haem propionate (Salemme 1977). In iso-1-cytochrome of mutants of the yeast Saccharomyces cerevisiae the equivalent tryptophan at position 64 is replaced by leucine, tyrosine and phenylalanine with concomitant loss of stability and biological activity (Schweingruber et al. 1978). Absence of tryptophan has been reported in the bacterial cytochromes c₅₅₃ of Desulfovibrio vulgaris Miyazaki strain (Yagi 1979), c₅₅₃ of D. vulgaris Hildenborough strain (Bruschi et al. 1972), iso-1-c₂ of Rhodospirillum fulvum and c₅₅₄ of Halotolerant micrococcus (Cookson et al. 1978). Hence the available evidence suggests that the requirement for tryptophan is not absolute and that this residue can be replaced in some cytochromes by other, probably hydrophobic, residues. This seems to be the case in Campylobacter cytochrome c₅₅₃.

Lysyl residues form 12% of the amino acid composition of Campylobacter cytochrome c₅₅₃. A variety of studies have implicated

lysine residues in the interaction of cytochrome c with its oxidoreductases. Studies on mitochondrial cytochrome c and cytochrome c_2 indicate that the interactions of cytochrome c with its physiological oxidoreductases are facilitated by a charge interaction between positively charged lysine residues of cytochrome c and complementarily charged sites on the oxidoreductases (Rieder and Bosshard 1978, 1980; Salemm 1976). Mitochondrial cytochrome c is a basic protein with a pI of approximately pH 10, corresponding to a preponderance of positive surface charges on the molecule. R. rubrum cytochrome c_2 exhibits a slightly acidic isoelectric point of 6.2 reflecting an approximately equal number of positive and negative charges on the molecular surface. Most bacterial cytochromes are acidic proteins and would be expected to carry a net negative charge at pH 7. Hence any assessment of the role of lysyl residues in the interaction of bacterial cytochromes c with their respective oxidoreductases must await the results of further studies.

iii. The Relationship of Cytochrome c_{553} to Chlorobia Cytochromes c_{555}

On the basis of amino acid sequence and three-dimensional structural similarities between cytochromes c of low spin and high midpoint potential, it has been proposed that, regardless of their origin or metabolic function, all these cytochromes belong to one evolutionary related family of protein molecules. It has been further argued that these cytochromes occupy similar positions in their respective electron transport chains; they are preceded by flavoproteins, quinones, iron sulphur proteins and cytochromes b and are followed by cytochrome oxidase or reaction centre bacteriochlorophyll. It was thus proposed that these cytochromes are related because the electron transport chains of which they are a part are also related; and the proposition that the electron transport chains of photosynthesis and

respiration have a common origin was put forward (Dickerson 1978, 1980). Dickerson et al. (1976) have argued that bacterial and eukaryotic respiration arose from the dual function cyclic photophosphorylation and respiratory electron transport chain of purple non-sulphur bacteria by loss of the photosynthetic capability.

Against the simple divergent picture proposed by Dickerson, Ambler et al. (1979a and b) have argued that anomalies in sequence similarities suggest that assimilation of genes for single functions or for whole metabolic pathways from other organisms might have taken place. In other words bacterial genomes are mosaics composed of genetic information from diverse sources with no simple divergent pattern of origin. Thus the cytochromes c_{551} and the cytochromes c' of Rps. gelatinosa and R. tenue all seem to be anomalously closer in sequence to proteins from organisms of other families including nonphotosynthetic ones than they are to proteins of other members of the Rhodospirillaceae.

While Dickerson (1978) concedes this as a disturbing observation, he argues lateral gene transfer of cytochrome c by itself is useless to the host organism unless accompanied by all the other components of the respiratory chain that interact with it. Dickerson also adds that, if such transfer of cytochrome c genes was common in bacteria, then polymorphism of the protein within one species would have been observed. The proposition is also made that tertiary structure is a more reliable index of similarity than primary structure (Dickerson 1978).

The arguments put forward by Dickerson et al. (1976) rest on the question of whether the cytochromes c are representative of their respective respiratory chains. It must be said however that not enough is known about the similarities between other components of these respiratory systems with regard to primary and tertiary structure to make confident assertions regarding the

relatedness of their electron transport systems. Moreover the possibility remains that the tertiary structure of the cytochromes c proteins might have arisen as the only particular and stable manner of folding of the polypeptide chain to generate a low spin high potential cytochrome c - that is that the present similarities we see are due to convergence.

The statistical method for sequence comparison from amino acid composition of Cornish-Bowden (1977, 1981) was utilized to compare the sequence similarity between Campylobacter cytochrome c₅₅₃ and a number of other cytochromes of similar lengths (table 14). These include cytochromes from a photosynthetic organism (Chlorobium), a denitrifying organism (Pseudomonas) and two sulphate reducing anaerobic organisms (the Desulfovibrios).

A pair of proteins fall in one of three categories according to the difference index $S\Delta n$:

1. the proteins are related if $S\Delta n < 0.42 N$
2. the result is inconclusive if

$$0.42 N < S\Delta n < 0.93 N$$

3. the proteins are unrelated if $S\Delta n > 0.93 N$

where N is the number of amino acid residues of the protein with the shorter sequence and

$$S\Delta n = \frac{1}{2} N_A^2 \sum \left(\frac{n_{iA}}{N_A} - \frac{n_{iB}}{N_B} \right)^2$$

where $S\Delta n$ is an estimate of loci at which the two sequences are different.

n_{iA} is the number of residues of the *i*th type in protein A, n_{iB} is the corresponding number in protein B and N_A and N_B are the total number of residues in A and B respectively.

In general $S\Delta n$ tends to exaggerate the amount of sequence difference; slightly if the proteins are related, but often grossly if they are not (Cornish-Bowden 1981).

Table 14: Sequence Comparison of Some Cytochromes c

Protein Pair	0.42N	SΔN	% difference in sequence	
			a	b
i. <u>Chlorobium thiosulphatophilum</u> PM c ₅₅₅ c <u>Chlorobium limicola</u> c ₅₅₅ c	35	55	64	46
ii. <u>Campylobacter sputorum</u> c ₅₅₃ d <u>Chlorobium limicola</u> c ₅₅₅	32	31	41	-
iii. <u>Campylobacter sputorum</u> c ₅₅₃ <u>Chlorobium thiosulphatophilum</u> PM c ₅₅₅	32	44	58	-
iv. <u>Pseudomonas aeruginosa</u> c ₅₅₁ e <u>Pseudomonas stutzeri</u> c ₅₅₁ e	34	14	17	33
v. <u>Chlorobium thiosulphatophilum</u> PM c ₅₅₅ <u>Pseudomonas aeruginosa</u> c ₅₅₁	34	82	100	85
vi. <u>Campylobacter sputorum</u> c ₅₅₃ <u>Pseudomonas aeruginosa</u> c ₅₅₁	32	47	62	-
vii. <u>Campylobacter sputorum</u> c ₅₅₃ <u>Pseudomonas stutzeri</u> c ₅₅₁	32	64	84	-
viii. <u>Campylobacter sputorum</u> c ₅₅₃ <u>Desulfovibrio desulfuricans</u> c ₅₅₃	32	63	83	-
ix. <u>Campylobacter sputorum</u> c ₅₅₃ <u>Desulfovibrio vulgaris</u> c ₅₅₃	32	72	95	-
x. <u>Desulfovibrio desulfuricans</u> c ₅₅₃ f <u>Desulfovibrio vulgaris</u> c ₅₅₃ f	34	42	51	-

References and Key Table 14:

(a) Calculated from S_AN. Aspartic and asparagine residues were treated as one amino acid for the purpose of calculation. Glutamine and glutamic acid residues were similarly treated.

(b) Calculated from sequence. Heads, tails and deletions were considered as one sequence difference each.

Amino acid composition were taken from:

(c) Van Beeumen and Ambler 1976

(d) This thesis

(e) Ambler and Wynn 1973

(f) Fauque et al. 1979.

Comparison of the figures obtained for the different proteins (table 14) leads to the following conclusions:

- a) by comparison to sequence difference obtained from actual sequence analysis the Cornish-Bowden statistical method exaggerates the difference between the two Chlorobia cytochromes c_{555} (table 14). The reverse effect is however observed for the two Pseudomonas cytochromes c_{551} .
- b) Comparative interpretation of the data is difficult. However taking the data at face value it does seem that Campylobacter cytochrome c_{553} is more similar in sequence to Chlorobium limicola c_{555} than the two Chlorobia cytochromes c_{555} are to each other; i.e. a cytochrome from a microaerophilic organism is more related to a cytochrome from an anaerobic photosynthetic organism than the latter is to another cytochrome from a photosynthetic organism from the same genus.

If anything such a conclusion tends to support the notions of Ambler et al. about lateral gene transfer between different bacterial species. While no firm conclusions can be drawn from the comparisons drawn above, the results do indicate that actual sequence comparison between cytochrome c_{553} and the two Chlorobia cytochromes c_{555} is well worth carrying out.

C. The Cytochromes c_{552}

i. The Nature of the Cytochromes c_{552}

While cytochrome c_{553} is clearly a low molecular weight soluble protein, the nature of the cytochromes c_{552} has not been unequivocally established. Penefsky et al. (1971) have defined a soluble preparation as that which remains in the supernatant fraction after centrifugation at 100,000 g for one or more hours. These workers however point out that procedures such as sonic irradiation, mechanical disruption or

extraction at alkaline pH may lead to dispersion of lipoprotein or to the formation of small membrane fragments which do not sediment readily and thus may appear to be soluble. They also point out that under conditions of prolonged centrifugation at high g values water soluble complexes of high molecular weight (i.e. of the order of several millions) may co-sediment with membrane fragments. The qualification of Penefsky et al. to their criterion of solubility indicate the difficulties in the determination of what is soluble and what is not.

The cytochromes c_{552} were extracted in 25 mM phosphate buffer/ 100 mM NaCl; were identified in the 165,000 g (15 hours) sedimentable material; and were observed to be retarded on Sephadex G200 and Sephacryl S300. Moreover catalase (molecular weight 232,000) was observed to sediment and form a pellet under the same centrifugation conditions. The pellet is rather easy to redissolve, simple shaking of the centrifuge tube being sufficient. While the crude pellet containing the cytochromes c_{552} was difficult to resuspend, the partially purified components sediment at the same g value, but by contrast were easily resuspended by simple shaking of the centrifuge tube. Addition of cholate to the extraction buffer (0.2% w/v) did not alter in any way the centrifugation and chromatographic behaviour of the cytochromes c_{552} .

A number of important points emerge from these observations. The cytochromes c_{552} do not necessarily have to be of very high molecular weight to sediment when centrifuged at 165,000 g for 15 hours. Other proteins present in the crude preparation may render the cytochromes c_{552} difficult to redissolve. High molecular weight material could be separated from the cytochromes c_{552} on Sepharose 4B (fig. 43). Hence the behaviour of the cytochromes on Sephadex and

Sephacryl might not be anomalous and may reflect molecular weights of approximately 200,000.

Thus we can tentatively conclude that the cytochromes c_{552} are soluble proteins. Because the cell breakage and extraction conditions were very mild and would not be expected to solubilise integral membrane proteins this would imply that the cytochromes c_{552} are not inserted in the membrane of the intact cell. Further evidence pertinent to the association of these cytochromes with membranes is necessary before a conclusive answer can be given. A number of experimental procedures would shed more light on the nature of the cytochromes c_{552} :

- a) acidification to pH 5 or addition of salts to a final concentration of 1-2%. This is a simple though by no means infallible step which may help to distinguish between proteins in true solution and suspended lipoprotein particles or lipoproteins. Lipoprotein material frequently will precipitate under such conditions and can be removed by low speed centrifugation (Penefsky et al. 1971).
- b) Destruction of the organic matters in the 165,000 g pellet and the partially purified cytochromes followed by determination of phosphate.
- c) Examination of material under the electron microscope. Vesicular membranes should be distinguished from large particles.
- d) Removal of the cell wall by lysozyme treatment followed by low speed centrifugation. Periplasmic material should appear in the low speed supernatant; membraneous material should be identified in the low speed pellet. In the case of Campylobacter Sputorum subspecies mucosalis, cytochrome c_{553} would be expected to be present in the supernatant while the cytochromes c_{552} would be present in the pellet only if they were membrane bound.

Were the cytochromes c_{552} to be proven as soluble, non-membraneous and periplasmic, then the question arises as to how Campylobacter sputorum subspecies mucosalis obtains energy from oxidative phosphorylation. The membraneous nature of electron transport components is a prerequisite to electron transport phosphorylation according to the chemiosmotic hypothesis.

A number of the above mentioned techniques are currently being utilized to further investigate the nature of the cytochrome c_{552} .

ii. Physicochemical Properties of the Cytochromes c_{552}

The striking feature of these cytochromes (c_{552} E_m -150 mV, c_{552} E_m -170 mV, and c_{552} E_m -290 mV) is their low midpoint potentials. Few cytochromes c with negative midpoint potentials have been identified. The properties of some low midpoint potential cytochromes c are summarized in table 15. Low midpoint potential cytochromes c have been identified in members of the sulphate reducing bacteria of the genus Desulfovibrio and comprise the cytochromes c_3 and one member of the cytochromes c_{553} . Cytochromes which are spectrally and potentiometrically similar to the cytochromes c_3 have also been isolated from photosynthetic bacteria. One of these, cytochrome $c_{551.5}$ of Chloropseudomonas ethylica, was eventually shown to arise from the Desulfuromonas acetoxidans member of this consortium (Probst et al. 1977). This cytochrome is a trihaem protein; while the cytochromes c_3 are tetrahaem proteins (Meyer et al. 1971; Yagi and Maruyama 1971). These cytochromes in general have a number of physicochemical properties which would serve to distinguish them from monohaem cytochromes c . The spectrum (fig. 4) is characterized by a shoulder at the short wavelengths side of the reduced Soret; the ratio δ_{red}/δ_{ox} is generally greater (table 15), and in the U.V. region the cytochromes c_3 show no maximum at 280 nm (fig. 4) (Le Gall et al. 1971; Der Vartanian et al. 1974). All of the cytochromes c_3 have low

Table 15: Some Properties of Low Potential Cytochromes c (Pseudomonas c₅₅₁ and Mitochondrial Cytochrome c are included for comparison)

Cytochrome	Absorption Maxima (nm)					Absorbance Ratios				References
	α	β	γ	δ_{red}/δ_{ox}	δ_{red}/α	M.W.	E_m	$\alpha/280$	Haem	
<u>D. Vulgaris c₃</u>	552	522	418	1.37	6.46	13,000	-205	2.95	4	Drucker et al. 1970 Horio and Kamen 1968 Meyer et al. 1971 Yagi and Maruyama 1971 Der Vartanian et al. 1978
<u>Rps. Saphaeroides c_{551.5}</u>	551.5	523	419	1.62	7.32	11,000	-254	1.7	2	Meyer et al. 1971
<u>D. Vulgaris (Miyazaki) c₅₅₃</u>	553	523	417	1.30	5.94	8,000	-260	1.23	1	Yagi 1979
<u>Desulfuromonas acetoxidans c_{551.5}</u>	551.5	522.5	418	1.63	6.44	9,800	-194	3.22-3.3	3	Fiechtner and Kassner 1979 Probst et al. 1977 Meyer et al. 1971 Shioi et al. 1972
<u>E. Coli</u>	552	523	420	1.40	4.75	36,000*	-194	1.03	4-6	Fujita and Sato 1966 Liu et al. 1981
<u>Campylobacter sputorum subspecies mucosalis c₅₅₂</u>	551.5	522.5	419	1.36	5.53	110,000* 200,000	-290 -170	0.24**	nd	This thesis
<u>Sephacryl Fraction I</u>	551.5	522.5	419	1.38	6.16	50,000	-150	0.28	nd	
<u>Sephacryl Fraction II</u>	551.5	522.5	419	1.38	6.16	50,000	-150	0.28	nd	

Table 15 contd.

Cytochrome	Absorption Maxima (nm)		Absorbance Ratios				M.W.	E_m	$\alpha/280$	Haem	References
	α	β	γ	δ_{red}/δ_{ox}	δ_{red}/α	δ_{red}/δ_{ox}					
<u>Pseudomonas aeruginosa</u> c ₅₅₁	551	521	416	1.4	4.9- 5.1		8,000	+286	1.25	1	Horio et al. 1960 Horio 1968a and b Ambler 1973
Mitochondrial Cytochrome c	550	521	416	1.22	5		12,200	+260	1.07- 1.25	1	Lemberg and Barret 1973 Dickerson and Timkovich 1975

*Molecular Weight on SDS

**Partially purified preparations

nd = not determined.

Fig. 15.

The physicochemical properties of Campylobacter cytochromes c_{552} are compared with those of other low midpoint potential cytochrome c . Only partial separation and purification of the cytochromes c_{552} was achieved. Hence the absorption maxima and absorbance ratios must be regarded as tentative. The molecular weights of the cytochromes c_{552} are those obtained on SDS electrophoresis and may not represent the molecular weights of the cytochromes in absence of denaturant.

spin character and contains sufficient histidine for it to provide the fifth and sixth ligands to the haem; indeed x-ray crystallographic studies have shown that this is the case (Haser et al. 1979). Hence the 695 nm band is absent (Drucker et al. 1970). There are indications from NMR, EPR as well as electrochemical titrations that the haems in cytochrome c_3 are not equivalent in their redox behaviour (Dobson et al. 1974; Xavier and Moura 1978; Dervartanian and Le Gall 1974; Dervartanian et al. 1978). Fiechtner and Kassner 1979 have presented evidence that two of the haems in cytochrome $c_{551.5}$ of Desulfuromonas acetoxidans were characterized by an E_m of -177 mV and the third by an E_m of -102 mV. The E_m of this cytochrome was originally reported as a single figure of -209 mV (Shioi et al. 1972) and -194 mV (Meyer et al. 1971). The cytochromes c_{553} isolated from the Desulfovibrios are distinguished from the cytochromes c_3 by their lower molecular weight, the single haem and except for cytochrome c_{553} of Desulfovibrio vulgaris (Miyazaki strain) by the less negative potential. This cytochrome has a midpoint potential of -260 mV; while cytochrome c_{553} of D. desulfuricans was reported to have a midpoint potential of $(50 \pm 20 \text{ mV})$ (Yagi 1979; Fauque et al. 1979).

The redox potentials of the cytochromes c_{552} of Campylobacter sputorum subspecies mucosalis (E_m -150 mV, -170 mV and -290 mV) are very low by comparison to many of the low spin c-type cytochromes so far isolated. These cytochromes share with the cytochromes c_3 the low midpoint potentials, the low spin character and the possible presence of a bis histidinyll adduct of the haem. However the absence of a shoulder in the spectra of the partially purified fractions (figs. 53 and 54) at the short wavelength side of the reduced Soret peak and the Soret red/Soret ox ratio distinguishes Campylobacter cytochromes c_{552} from the cytochromes c_3 . The molecular weights of

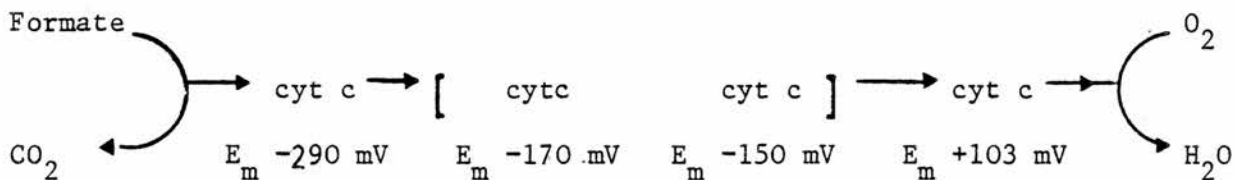
the cytochromes c_{552} are also considerably greater than those reported for the cytochromes c_3 (table 15).

The molecular weight (table 15) distinguishes Campylobacter cytochromes c_{552} from D. vulgaris (Miyazaki) cytochrome c_{553} and from Desulfuromonas acetoxidans cytochrome $c_{551.5}$ as well as from E. Coli cytochrome c_{552} of Fujita (1966). The latter cytochrome was reported to have an E_m of -194 mV to -220 mV. More recently (Liu et al. 1981) the cytochrome was reported to contain 4-6 haems/molecule and no evidence was found for the presence of subunits. Fujita and Sato 1966 have reported that the cytochrome contains 10 haems/molecule. The cytochrome was also shown to exhibit nitrite reductase activity (Liu et al. 1981).

Thus the available data do not indicate any clear relative to Campylobacter sputorum subspecies mucosalis cytochromes c_{552} . These cytochromes may represent a novel group of cytochromes and may be unique to the Campylobacters.

D. The Electron Transport Chain of Campylobacter Sputorum Subspecies Mucosalis

Redox potentiometric titrations of the crude extracts of Campylobacter sputorum subspecies mucosalis were carried out in the presence of redox mediators. These short circuit the whole respiratory system making all cytochromes amenable to reduction. However formate has been observed to produce total reduction of all cytochromes c in cell sonicates of the organism under anaerobic conditions (J. Leaver unpublished observations). The redox potentiometric data are hence consistent with electron flow through the respiratory chain of Campylobacter sputorum subspecies mucosalis in the following manner:



As has been emphasized in the introduction the characterization of the electron transport systems of Campylobacters is still at a very preliminary stage. It can however be said that the microaerophilic respiratory chain of Campylobacter sputorum subspecies mucosalis shows similarity to those reported for other Campylobacters. Cytochromes c, partially reducible by ascorbate, have been identified in two members of the genus Campylobacter; thus suggesting the presence of both low and high midpoint potential forms of the cytochrome (Harvey and Lascelles 1980; Niekus et al. 1980a). The high potential cytochromes c were proposed to participate in electron transport at the oxidising ends of the respiratory chains of Campylobacter fetus subspecies intestinalis (Harvey and Lascelles 1980) and Campylobacter sputorum subspecies bubulus (Niekus et al. 1980a). In the latter organism no role was suggested for the low midpoint potentials cytochromes c identified. In the former organism a bypass (fig. 8b) at the reducing end of the respiratory chain involving a low midpoint potential cytochrome c was suggested (Harvey and Lascelles 1980).

b-type cytochromes were shown to participate at the reducing ends of the electron transport chains of Campylobacter fetus subspecies intestinalis (Harvey and Lascelles 1980), and of Campylobacter species of Laanbroek et al. 1978 (fig. 8b). No role was proposed for b-type cytochromes identified in Campylobacter sputorum subspecies bubulus (Niekus et al. 1980a). Protohaem IX indicative of the presence of b-type cytochromes has been identified in relatively minor amounts in Campylobacter sputorum subspecies mucosalis (Lawson et al. 1981).

The function of the corresponding b-type cytochrome remains to be determined. It should be emphasized however that it is present in such small amounts relative to the c-type cytochromes that it is only observed as a small shoulder at 560 nm on the α -peak of c-type cytochromes which occur in the low speed pellet material.

Campylobacter sputorum subspecies mucosalis was grown under hydrogen; cytochrome reduction by formate suggests the presence of a constitutive formate dehydrogenase. The enzyme has been identified in a number of organisms under different growth conditions. It has been reported for E. Coli (Enoch and Lester 1975), for Vibrio succinogenes (Krüger 1979), and for Campylobacter fetus subspecies intestinalis (Harvey and Lascelles 1980). In all these cases a b-type cytochrome was reported to be the electron acceptor for the dehydrogenase. The data of Harvey and Lascelles 1980 are however also consistent with a bypass involving a low potential c-type cytochrome only (fig. 8b). The midpoint potential of Campylobacter sputorum subspecies mucosalis cytochrome c_{552} (E_m -290 mV) would be consistent with a role as the immediate electron acceptor for formate dehydrogenase. Only one other cytochrome c - c_{553} of D. vulgaris (Miyazaki) E_m -260 mV - is reported to function in a similar capacity (Yagi 1979). It is noteworthy however that the formate dehydrogenase of D. vulgaris (Hildenborough) could reduce both cytochrome c_3 and cytochrome c_{553} from the same organism. The midpoint potential of the latter is not known exactly but is reported to be less negative than that of cytochrome c_3 ; while the formate dehydrogenase from D. gigas would reduce cytochromes c_3 of either D. gigas or D. vulgaris (Dervartanian et al. 1974). Thus the specificity of formate dehydrogenase of these organisms is still doubtful.

The midpoint potential of Campylobacter cytochrome c_{552} (E_m -290 mV) also makes it a likely candidate as an electron acceptor for reducing equivalents from hydrogenase. Cytochromes c of similar midpoint potentials - the cytochromes c_3 - have been isolated from the sulphate reducing bacteria. Campylobacter sputorum subspecies mucosalis was grown under hydrogen; and at least one member of the genus viz Campylobacter species of Laanbroek et al. 1978 has been shown to be able to reduce sulphite, thiosulphate and elemental sulphur but not sulphate. Hydrogenase was reported to a constitutive enzyme in the organism (Laanbroek et al. 1978). In this respect this member of the genus Campylobacter shows similarities to Desulfovibrio vulgaris which can grow on hydrogen or formate plus sulphate with acetate and carbon dioxide as sole carbon sources (Badziong et al. 1978). The ability for growth under anaerobic conditions in the presence of sulphur compounds as terminal electron acceptors has so far not been demonstrated for Campylobacter sputorum subspecies mucosalis.

Campylobacter sputorum subspecies mucosalis can grow in the presence of nitrate and fumarate as terminal electron acceptors. Two important results emerge from potentiometric titrations of cytochromes c of the organism grown in the presence of nitrate or fumarate (M. Symmons - personal communication):

- a) under fumarate respiration there is substantial loss of cytochrome c_{553} . In addition the cells produce less of the sedimenting high potential species relative to low potential cytochromes.
- b) Under nitrate respiration very low contents of c-type cytochromes are identified (2 nmol/g cells vs approximately 100 nmol/g cells for microaerophillically grown cells). Moreover the difference spectrum of the low speed pellet shows a prominent contribution of b-type cytochromes.

The involvement of cytochrome c in electron transport during fumarate respiration has so far not been reported. One of the best studied bacterial respiratory chains - that of Vibrio succinogenes - is reported to contain b-type cytochromes only (Krøger et al. 1976). C-type cytochromes identified in Vibrio succinogenes were proposed to be involved in electron transport to an acceptor of a more positive redox potential - oxygen and/or nitrate (Krøger et al. 1976). A similar proposition was made for the c-type cytochromes of Campylobacter species of Laanbroek et al. 1978. By contrast the observations of M. Symmons clearly implicate low potential cytochromes c in respiration to fumarate in Campylobacter sputorum subspecies mucosalis. On the other hand a dramatic suppression of c-type cytochromes appear to take place in the organism during nitrate respiration. As a result the b-type cytochromes become prominent. In this respect the organism shows some similarity to E. Coli. It is noteworthy however that in E. Coli the aerobic respiratory chain is reported to be functional during nitrate respiration (Haddock and Jones 1977). The redox potentiometric data of M. Symmons for fumarate respiration suggest a specific role for cytochrome c_{553} and the high potential 165,000 g sedimentable cytochrome c in the microaerophilic respiratory chain of Campylobacter sputorum subspecies mucosalis. Overall the available evidence indicates the ability of the organism to regulate the composition of its respiratory chain under different growth conditions.

Two points need to be emphasized before this treatment of the respiratory chain of Campylobacter sputorum subspecies mucosalis is concluded:

a) formate has been observed to reduce all cytochromes c of the microaerophilic respiratory chain. However branching of the microaerophilic respiratory chain of Campylobacter sputorum subspecies mucosalis

cannot be excluded; and the points of entry and/or exit of reducing equivalents from other electron chains such as NADH may not necessarily be those of reducing equivalent from formate.

b) Fumarate and nitrate reductases would be induced in the respiratory chain during fumarate and nitrate respiration. Lawson et al. 1981 have reported the possible presence of cytochrome d in Campylobacter sputorum subspecies mucosalis grown microaerophillically. Enhanced synthesis of cytochrome d has been observed in a number of organisms under conditions of oxygen deprivation (Jones 1977). The presence of cytochrome d would be consistent with the microaerophilic nature of Campylobacter sputorum subspecies mucosalis. Such an oxidase was suggested to have high turnover numbers or exhibit increased affinities for molecular oxygen (Jones 1977).

Contrary to the observations of Lawson et al. 1981 M. Symmons (personal communication) could not detect cytochrome d in Campylobacter sputorum subspecies mucosalis. The cytochrome was however detectable in Vibrio succinogenes grown under the same microaerophilic conditions. Hence the terminal oxidase of the microaerophilic respiratory chain of the former organism remains unidentified.

E. Concluding Remarks

Considerable progress has been made towards the realization of the objectives outlined in chapter I. The presence of cytochrome c_{553} of Campylobacter sputorum subspecies mucosalis was predicted from redox potentiometry of the crude fractions and the cytochrome was purified and characterized. The redox potentiometric data were however indicative of the presence of a second high midpoint potential cytochrome c which sediments in the 165,000 g pellet and which masks the asymmetry of the α -peak of cytochrome c_{553} in the crude cell extract.

No further evidence for the presence of this cytochrome was obtained. It may be that this cytochrome is that component with a less negative potential than cytochrome c_{552} (E_m -150 mV) in Sephacryl Fraction II. This is however speculative since not enough data was obtained to measure its midpoint potential. Only partial separation of cytochrome c_{552} (E_m -150 mV) has been achieved. Sephacryl Fraction II was enriched in the cytochrome to the extent of 66% and all indications are that complete separation of this component in a potentiometrically homogeneous form is feasible. The separation of the two other cytochromes (c_{552} E_m -170 mV and c_{552} E_m -290 mV) of Sephacryl Fraction I was not possible by the techniques employed in this work. The possibility of separation by other chromatographic techniques remains to be explored.

An important point which emerges from these studies is the difficulties encountered in the resolution of components with similar midpoint potentials during redox titrations of the crude preparations. Campylobacter cytochrome c_{552} E_m -150 mV and c_{552} E_m -170 mV were not mathematically resolved during titrations of the crude preparations. In fact values of -125 mV were reported. Despite this discrepancy the comparison between potentiometric characterization of the whole system and of the purified components shows good agreement and reflects favourably on the reliability of the resolution of the redox potentiometric curves. However the problem of resolution clearly indicate the desirability of:

- a) the physical separation of the potentiometrically identified species and;
- b) comparison of the midpoint potentials of the potentiometrically homogeneous species with values predicted from redox titrations of the crude cell extracts.

The results presented in this work represent only partial characterization of the electron transport chain of Campylobacter sputorum subspecies mucosalis. Interpretation of the data was based on the assumption that the cytochromes c present in the crude cell extract were representative of the electron transport chain of the organism. 40% of the total cytochrome c in cells of the organism was observed to sediment with the cell debris at 20,000 g (30 minutes) - indicating a membraneous nature. It has been known for some time that membrane bound cytochromes c are different from soluble cytochromes c. This has been demonstrated for mitochondrial cytochromes c_1 and c and for the membrane bound and soluble cytochrome c_{551} of Pseudomonas (King 1978; Wood and Willey 1980). It is not however clear whether Wood and Willey 1980 have examined the cell debris (10,000 g for 10 minutes pellet) for associated cytochromes. A similar observation is pertinent to the characterization of cytochromes b of E. Coli (Reid and Ingledew 1978). Also in the latter case, and in the case of Hendler et al. 1975 and Pudek and Bragg 1976, no information was given as to the properties of soluble cytochromes - if any - in the 100,000 g supernatant. It is therefore worthwhile bearing in mind that the organization of bacterial electron transport chains does not survive cell breakage and that the examination of a number of fractions is necessary for complete characterization of the electron transport chain. By contrast the advantage of isolating the mitochondria as an intact structural entity is immediately obvious, and has enabled Dutton et al. 1970 to potentiometrically characterize all the cytochromes of the mitochondrial electron transport chain.

Until such time as the midpoint potentials of the cytochromes c associated with the low speed pellet has been determined, the proposed electron transport chain for Campylobacter sputorum subspecies mucosalis

should be regarded as tentative and preliminary. Special techniques involving dual wavelength spectrophotometry are needed to characterize these cytochromes. Moreover further studies are needed for full characterization of the electron transport chain of Campylobacter sputorum subspecies mucosalis. Redox potentiometry as such can only provide a framework for further studies and provides no information on branching of the respiratory chain - a phenomenon frequently observed in bacteria - Jones 1977. Information available from redox potentiometry would have to be supplemented with studies of the electron transport chain in the presence and absence of different electron transport inhibitors and the state of reduction of the cytochromes with different electron donors. The terminal oxidases need to be identified, and the role of cytochrome b in the microaerophilic respiratory chain defined. This study is part of an overall attempt to gain some insight into the energy metabolism of an important organism. It is hoped that such studies might lead to full characterization of the electron transport chain of Campylobacter sputorum subspecies mucosalis, while investigation of the essential requirements for optimum growth of the organism might provide some information on the factors which govern the metabolic relationship of the organism to its host cell in "vivo".

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The *c*-type cytochromes of *Campylobacter sputorum* ssp. *mucosalis*

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Received 15 February 1982

Accepted 9 March 1982

1. INTRODUCTION

Members of the genus *Campylobacter* have been implicated in a wide variety of infections in man and domestic animals [1].

Campylobacter sputorum ssp. *mucosalis* was isolated from pig adenomatous intestinal tissue by Lawson and co-workers [2]. This and other *Campylobacter* species can grow micro-aerophilically with hydrogen or formate as electron donor and can also grow anaerobically with fumarate as electron acceptor [3–5].

In these respects and in morphology they resemble *Vibrio succinogenes*, a rumen bacterium which has one of the best characterised bacterial electron transport systems [6]. However, in *V. succinogenes* *b*-type cytochromes function in electron transport between formate and fumarate while in *C. sputorum* ssp. *mucosalis* large amounts of *c*-type cytochrome and very little *b*-type cytochrome were found [3].

This paper describes the potentiometric and physical resolution of the *c*-type cytochromes of *C. sputorum* ssp. *mucosalis* grown micro-aerophilically in the presence of hydrogen. We emphasise the distinctive presence of very low potential *c*-type cytochromes and compare these with cytochromes of other organisms.

2. MATERIALS AND METHODS

2.1. Organism and culture

C. sputorum ssp. *mucosalis* was an isolate from adenomatous pig intestinal tissue kindly provided by Dr. G.H.K. Lawson, University of Edinburgh. The organism was grown micro-aerophilically on 'Columbia' blood agar plates for 48–72 h in an atmosphere of 4% O₂, 5% CO₂, 14% N₂, 77% H₂ and harvested by scraping from the agar surface. The cells were washed in 5 mM sodium phosphate buffer pH 7.0 and stored at –40°C.

2.2. Preparation of total extract, and soluble and sedimentable fractions

All operations were conducted at 4°C. Cells were disrupted by freezing and thawing twice, and homogenised in 2 vols. of 25 mM sodium phosphate pH 7.0 containing 100 mM NaCl. The broken cell suspension was passed through a French pressure cell at 12000 psi and then centrifuged at 10000 × *g* for 30 min. The supernatant, subsequently described as the total extract, was used for redox potentiometry or was subjected to centrifugation at 160000 × *g* for 15 h to yield a glassy red pellet, described as the sedimentable fraction, and a red supernatant described as the soluble fraction.

2.3. Redox potentiometry

The total extract, and the soluble and sedimentable fractions, were examined by simultaneous redox potentiometry and spectrophotometry based on the methods of Dutton [7].

The ambient redox potential was monitored by a Pt-Ag/AgCl combination electrode (Russell pH Ltd., Auchtermuchty, Fife, U.K.). The redox potential of the Ag/AgCl half cell was measured against standard Fe-EDTA mixtures [8] and a value of 195 ± 5 mV was obtained. This value (which is approx. 30 mV less positive than the accepted value for such an electrode [7]) was used to calculate the redox potential of solutions vs. the normal hydrogen electrode.

Solutions contained the redox mediators, benzyl viologen, flavin mononucleotide, hydroxynaphthoquinone sulphonate, duroquinone, phenazine methosulphate, phenazine ethosulphate and diaminodurool (all 10 μ M) and 20 μ M Fe^{3+} , 0.4 mM EDTA.

Spectra were recorded in a Unicam Sp1800 spectrophotometer modified to accept a cuvette which could be stirred and supplied with argon. Argon was passed over the cuvette contents for 60 min before titration to ensure anaerobic conditions. Before each measurement the absorbance at 544 nm, an isosbestic point for the cytochromes, was adjusted to a fixed value.

The titration data were plotted in the form E_h vs. $\log(\text{total ox.})/(\text{total red.})$ (Fig. 2, a-c) and the complex curves were resolved arithmetically using the positions of the sigmoidal regions as indicators of the relative contribution of each species to the total absorbance. Once the contribution of each component had been resolved, a composite theoretical curve could be generated and compared to the original data (Fig. 2, a-c).

3. RESULTS

3.1. The use of formate as reductant

In the cases of the total extract and the sedimentable fraction, addition of 60 mM sodium formate could be used as a reductant, indicating

the presence of formate dehydrogenase. The redox mediators act to short circuit the electron transport chain so that electrons from formate are freely available to all redox centres in the system. However, NADH and NADH-cytochrome *c* reductase were used to initiate titrations and obtain stable redox potentials before formate was used. This was necessary because the initiation of titrations with formate resulted in a drift of redox potential to more positive values, perhaps due to hydrogen peroxide production from residual O_2 by formate dehydrogenase [9]. NADH and NADH-cytochrome *c* reductase were used as the reducing system throughout the titration of the soluble fraction because of the low residual activity of formate dehydrogenase.

In crude extracts, sodium dithionite could not be used as a reductant due to a very rapid drift of ambient redox potential to more positive values. This may indicate the presence of an oxidase system using a dithionite oxidation product as an electron acceptor.

3.2. Redox titration of total extract

The titration curve (Fig. 2a) could be resolved into three components with $E_{m,1} + 86$ mV (31%), $E_{m,2} - 130$ mV (38%) and $E_{m,3} - 320$ mV (31%) (Fig. 3a). These contributions were used to construct a theoretical curve (Fig. 2a) which agreed well with the experimental data except in the region of most positive redox potential. This may indicate the presence of more than one cytochrome of positive mid-point potential and this is supported by the two further titrations described below.

3.3. Redox titration of soluble fraction

Again the titration curve (Fig. 2b) could be resolved into three components (Fig. 3b) with $E_{m,1} + 92$ mV (54%), $E_{m,2} - 135$ mV (27%) and $E_{m,3} - 300$ mV (19%). The component $E_{m,1}$ showed a markedly asymmetric α -peak at 553 nm (Fig. 1) and this component must contribute to the species with $E_{m,1}$ of 86 mV observed in titration of the total extract. The lack of asymmetry of the α -peak in the total extract is probably due to the presence

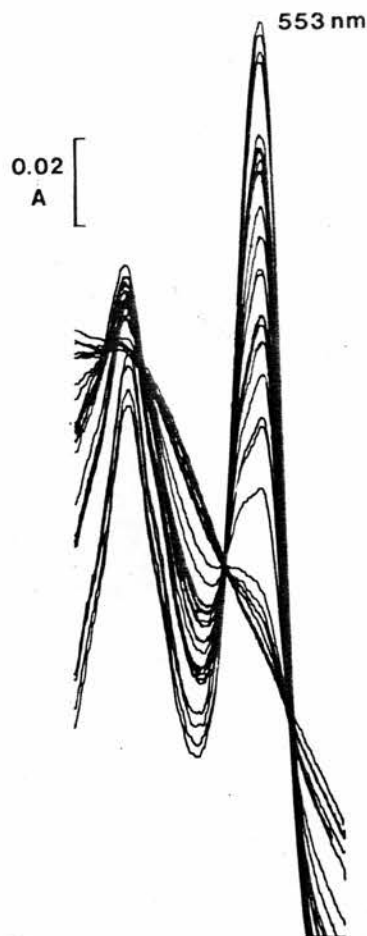


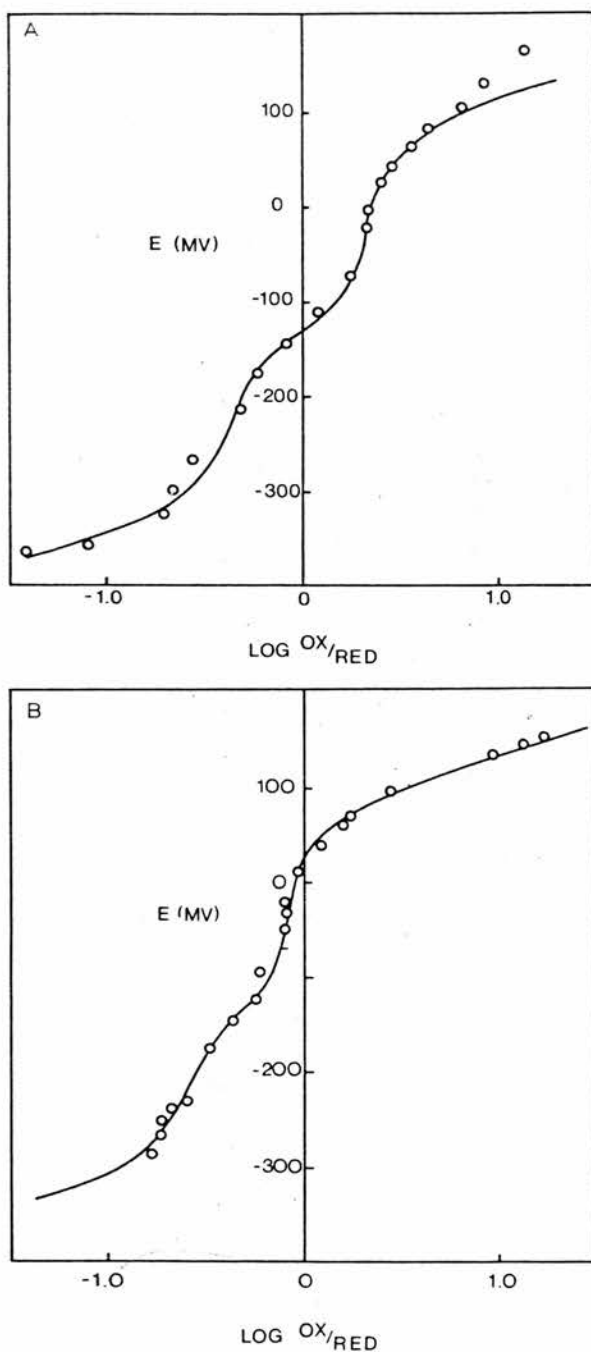
Fig. 1. Potentiometric titration of the *c*-type cytochromes of the soluble fraction. Spectra were recorded after establishment of stable redox potentials (not included). Before each scan the absorbance at 544 nm was adjusted to an arbitrary fixed value. The degree of reduction as a function of redox potential is plotted in Fig. 2a.

of a second species titrating at similar redox potentials and with a symmetric α -peak at 552 nm.

We therefore propose that the cytochrome with asymmetric α -peak is a soluble species, periplasmic or cytoplasmic in location and constituting approx. 15% of the total extracted cytochrome (Fig. 4). The persistence of species titrating at negative redox potentials in this 'soluble' fraction does not necessarily indicate partial solubilisation of these species because the $160000 \times g$ supernatant was graded in colour just above the pellet and therefore still contained partially sedimented material.

3.4. Redox titration of the sedimentable fraction

The titration curve (Fig. 2c) was resolved into three components (Fig. 3c) with $E_{m,1} + 80$ mV (21%), $E_{m,2} - 125$ mV (37%), and $E_{m,3} - 320$ mV



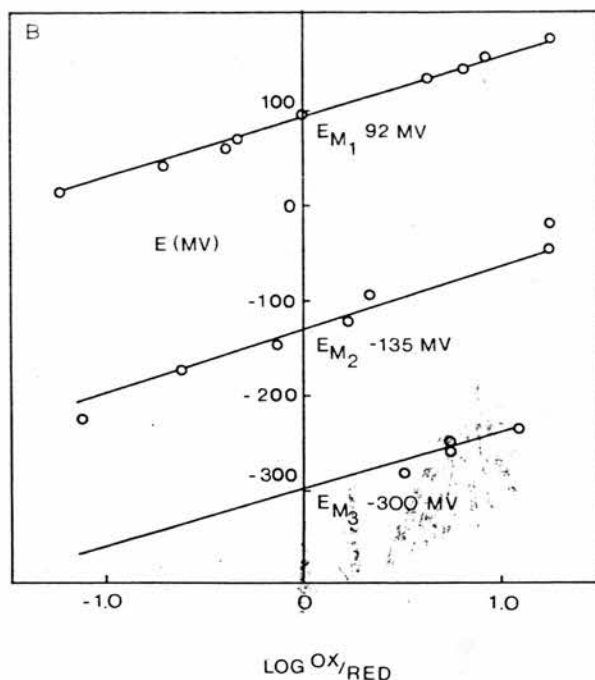
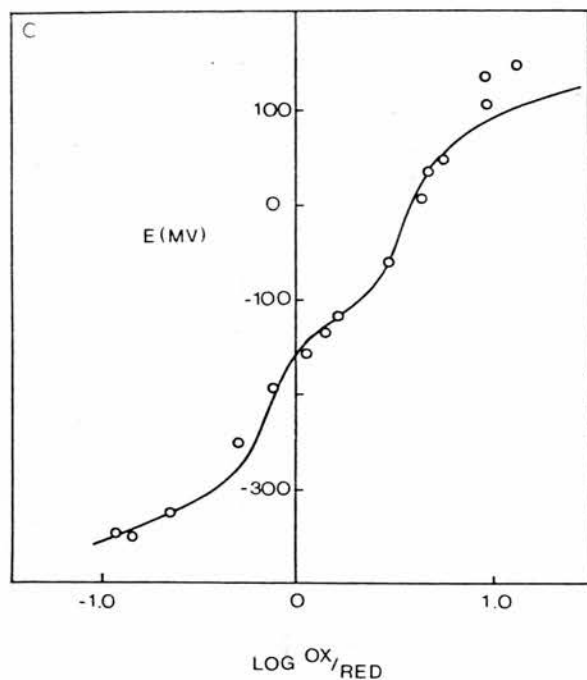


Fig. 2. Potentiometric titration of the *c*-type cytochromes of (a) total extract; (b) soluble fraction, and (c) sedimentable fraction. Experimental points are from spectra of the type shown in Fig. 1 along with corresponding redox potentials. Lines are theoretical curves constructed on the basis of the mid-point potential values and the relative proportions of each species deduced from the experimental data.

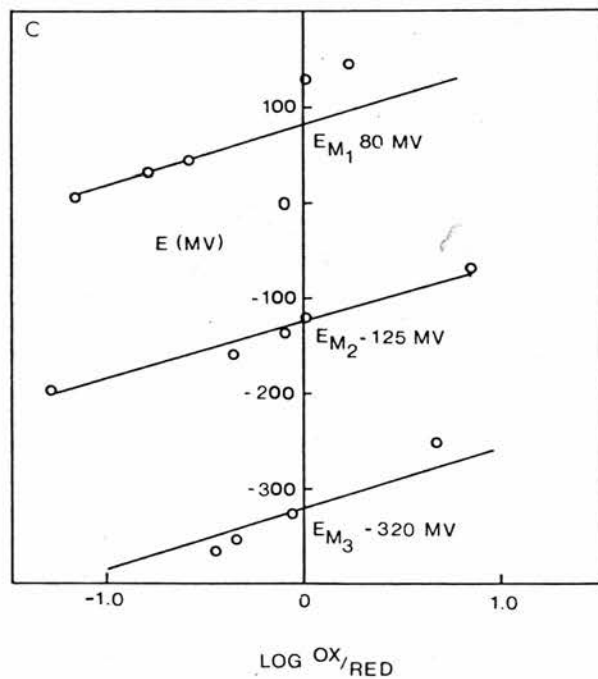
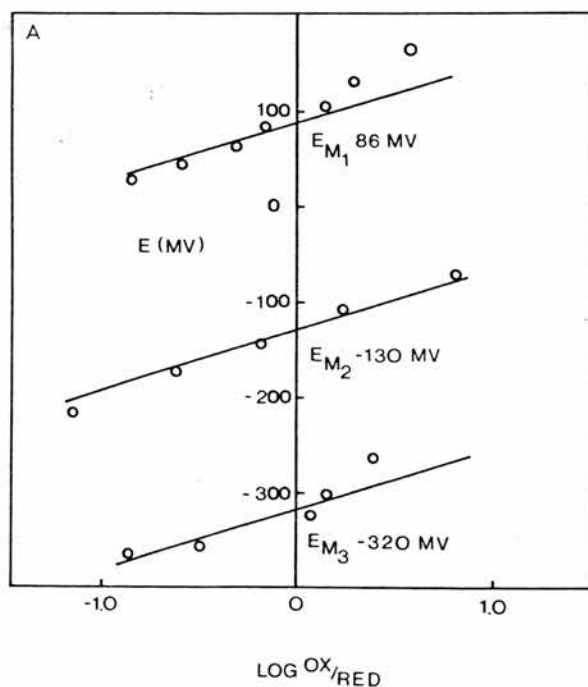


Fig. 3. Resolution of the potentiometric titration of the *c*-type cytochromes of (a) total extract; (b) soluble fraction, and (c) sedimentable fraction. The components contributing to the complex curves of Fig. 2 were separated on the basis of the inflexion points and recalculation of log ox/red. Lines are slopes of 60 mV.

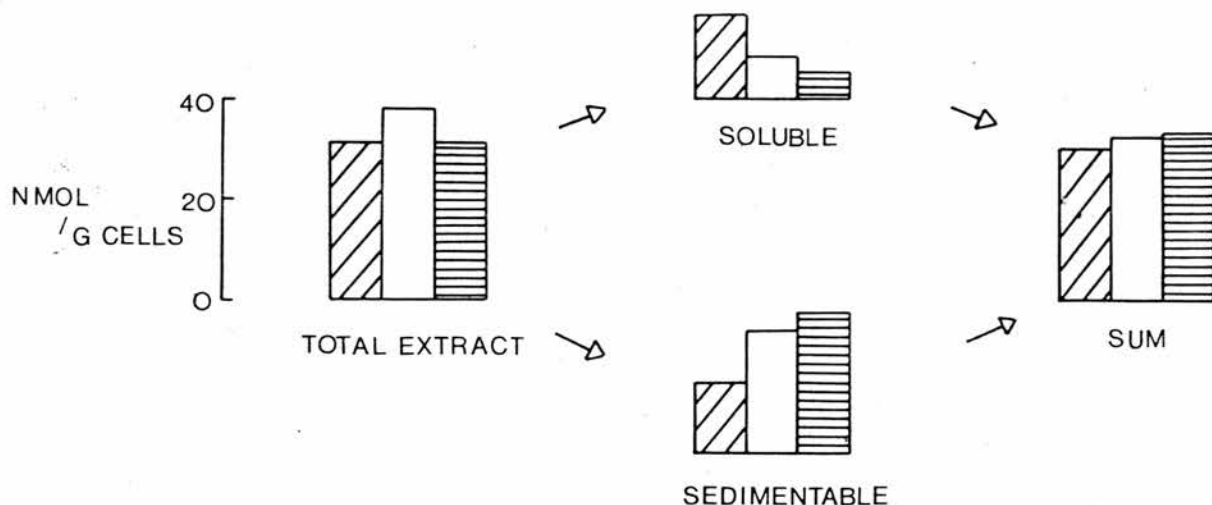


Fig. 4. Proportions of the *c*-type cytochrome redox species. The amounts of each redox species observed for an extract of 1 g wet weight of cells are shown. Proportions are deduced from the positions of inflexion points in the curves shown in Fig. 2. The concentrations of each cytochrome present was calculated assuming an extinction coefficient at 20 mM/cm for ($A_{552\text{red}} - A_{552\text{ox}}$). ▨, component E_{m1} ; □, component E_{m2} ; ▩, component E_{m3} .

(42%). When combined with the amounts of the components found in the soluble fraction, relative proportions very similar to those found in the total extract were obtained (Fig. 4). The component E_{m1} in this titration showed a symmetric α -peak at 552 nm and we propose that this is distinct from the component of positive mid-point potential observed in the soluble fraction. The presence of this sedimentable species with a symmetric α -peak complicates the titration of the total extract and obscures the presence of the cytochrome with asymmetric α -peak clearly observed in the soluble fraction (Fig. 1).

4. DISCUSSION

The potentiometric titrations revealed the presence of at least 3 *c*-type cytochromes in extracts of *C. sputorum* ssp. *mucosalis*. Two sedimentable *c*-type cytochromes, probably membrane-associated, have mid-point potentials of -135 mV and -300 mV. A soluble *c*-type cytochrome, with a mid-point potential of $+86$ mV, has an asymmetric α -peak. However, in the total extract this asymmetry is apparently masked by the presence of small amounts of a fourth cytochrome which is sedimen-

table and has a similar mid-point potential, but a symmetric α -peak.

The low potential *c*-type cytochromes are particularly interesting since relatively few such cytochromes are known, the best characterised being the cytochromes c_3 of the sulphate-reducing bacteria [10]. However, the apparent membrane association and spectroscopic features of the *Campylobacter* cytochromes argue against any close relationship to the cytochrome c_3 [2].

Other species of *Campylobacter* contain *c*-type cytochromes which are only partially reducible by ascorbate [4,11,12]. Thus the occurrence of both low- and high-potential *c*-type cytochromes may be a characteristic feature of the genus. Indeed this may be a feature which the *Campylobacters* share with *Vibrio succinogenes* where 2 *c*-type cytochromes are present with mid-point potentials of -160 mV and $+70$ mV [13].

In *C. sputorum* ssp. *mucosalis* all the *c*-type cytochromes are reducible with formate or hydrogen [3] and may therefore be part of an electron transport system from these donors to oxygen. However, the organism also grows anaerobically if nitrate or fumarate are supplied and in this respect it resembles *E. coli* and *Vibrio succinogenes*. In the latter, the two *c*-type cytochromes are apparently

not part of the formate-fumarate pathway which appears to involve *b*-type cytochromes only. In *E. coli* a cytochrome *c*-552 with mid-point potential of approx. -200 mV is found in anaerobically grown cells [14].

We are currently investigating the role of the different potentiometric cytochrome species by selective reduction and oxidation, and by growth under different respiratory conditions.

ACKNOWLEDGEMENTS

Our thanks to Elizabeth McNeillie who was involved in some of the preliminary stages of the work presented here, to Dr. Gordon Lawson for providing cultures and for valuable advice, and to the Wellcome Trust for financial support.

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